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BIOLOGICAL AND PHYSICAL PROPERTIES OF THE HEMOTOXIN OF STREPTOCOCCI.

By PERCY D. MEADER, Ph.D., and GEORGE H. ROBINSON, Ph.D.

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(Received for publication, June 17, 1920.)

The isolation of hemolytic streptococci from many different pathological lesions demonstrates their importance as pathogenic organisms. Various investigators point out two types of these organisms, one of which produces hemolysis only on blood agar, while the other produces also a hemolytic substance, or hemotoxin, in bouillon. Whether or not all laking of erythrocytes as exhibited on blood agar plates by different strains is the result of the same reaction is not clear. However, the ability to produce hemotoxin in bouillon seems to be a definite characteristic of certain strains of streptococci. Since hemolytic power is a biological function of most virulent streptococci, it is of interest to note the nature of the hemotoxin.

Frequent attempts have been made to separate hemotoxin from the organisms by filtration. The matter of filtration is of the first importance in showing whether the hemotoxin is in solution or is contained within the bacterial cells. Aronson (1902) states that hemotoxin can pass through a filter. Besredka (1901), using a Chamberland filter, noted a relation between the rate of filtration and the hemolytic strength of the filtrate and suggests that perhaps the active substance is retained in the pores of the filter. Ruediger (1903) recommends that the filter should be carefully selected, as too fine a filter removes the hemolytic property. Von Hellens (1913) and Nakayama (1919) were able to obtain hemolytic filtrates only with considerable loss of potency during the procedure. Maasen filters were used in their experiments. M'Leod (1911–12) and Braun (1912) were able to secure hemolytic filtrates by the use of Maasen filters. Lyall (1914) was not able to obtain hemolytic filtrates by the use of coarse Berkefeld filters.

Another method of separating the bacterial cells from the hemotoxin is by centrifugation. Levin (1903) reported that there was nearly 1,000 times as much hemotoxin in the sediment of a centrifuged tube of medium as in the filtrate of

the same fluid. A comparison of the sediment and the supernatant fluid after centrifugation would seem to have more value, however. Schlesinger (1903) concentrated the streptococci by centrifuging and found that hemotoxin was liberated by crushing the cells.

Little is recorded regarding the chemical nature of the hemotoxin. Ruediger (1903) states that it is composed of a toxophore and haptophore group loosely bound together. Von Hellens (1913) found hemotoxin soluble in ether and extracted it by this method. Von Lingelsheim (1912) reports that hemotoxin is of the nature of an enzyme. Lyall (1914) is of the opinion that it is not an enzyme because the hemolytic power of his cultures was destroyed by exposure to chloroform and toluene, reagents not destructive to enzymes.

The culture medium used by different workers for the production of hemotoxin varies widely. M'Leod (1911–12) found that the addition of 15 to 20 per cent of horse serum to bouillon produced the most powerful hemotoxin. Nakayama (1919) added 50 per cent or more horse serum to broth. Von Hellens (1913), on the other hand, found that the addition of horse serum in amounts of more than 40 per cent tended to decrease the amount of hemotoxin produced. Lyall (1914) used a calcium carbonate broth to which 16 per cent ascitic fluid was added.

Owing to the contradictory results reported in the literature a repetition of much of the work on this subject seemed necessary. The object of the work reported in this paper was to determine if possible something of the nature of the hemotoxin by means of a study of its filterability, the effect of adsorptive agents, the effect of centrifugation and shaking, and the basic substances from which it is produced.

In all the work a standard culture medium was used consisting of bouillon with 1 per cent of Difco peptone prepared according to the method of Huntoon (1918), to which were added 3 drops of normal horse serum per 5 cc. of the medium. The strains of streptococci used were obtained from cases of empyema and were supplied by the Army Medical School. Hemolytic tests were accomplished by adding to 0.5 cc. amounts of the proper dilutions of the test substance 0.5 cc. of 5 per cent washed rabbit corpuscles. The tubes were then incubated for 1 hour in the water bath at 37°C. The relative susceptibility of the corpuscles from different animal species was not determined. It was found that the hemotoxin of the streptococcus was not specific for red blood cells but had the power of destroying various types of muscular and glandular tissue.

Effect of Filtration and Adsorption on the Hemotoxin.

The effect of filtration on hemotoxin may be determined by a few simple experiments. It was found that there was considerable loss during passage of bouillon through Mandler filters. If the hemolytic substance is held in the pores of the filter, reversal of the filter and washing with the filtrate should restore the hemolytic titer. No hemotoxin was recovered by this method. The rate of filtration was observed to have considerable effect upon the amount of hemotoxin in the filtrate; *i.e.*, the more rapidly the bouillon was forced through the filter, the greater the amount of hemotoxin recovered. As a control on this experiment a broken filter was finely pulverized in a mortar and 2 gm. were added to each of two tubes containing 5 cc. of

TABLE I.

Treatment.	0.5 cc.	0.25 cc.	0.12 cc.	0.06 cc.	0.03 cc.	0.015 cc.
Untreated bouillon culture	++	++	++	++	++	+
Rapid filtration	++	++	+	−	−	−
Slow "	−	−	−	−	−	−
Reversal of the filter	−	−	−	−	−	−
Rapid adsorption	++	++	++	++	−	−
30 min. "	−	−	−	−	−	−
Addition of calcium chloride	+	−	−	−	−	−

++ designates complete hemolysis; +, partial hemolysis; −, no hemolysis.

hemolytic bouillon culture. These were shaken to insure an even distribution of the pulverized material throughout the bouillon. One was immediately centrifuged and the other allowed to stand for 30 minutes before centrifugation. The first tube showed slight diminution of hemolytic titer, while the second showed no hemotoxin present in the liquid.

The addition of calcium chloride to bouillon causes a precipitate which may act as an adsorptive agent. Barium carbonate will also remove the greater part of the hemotoxin from bouillon.

From Table I the similarity in action between rapid filtration and adsorption can be observed. It is especially significant, since the same material was used in both processes. The failure to recover hemotoxin by reversal of the filter and passage of the filtrate back

through the filter indicates either the destruction of hemotoxin or its firm combination with the filter material. If infusion bouillon is passed through a diatomaceous filter before inoculation with hemolytic streptococci good growth results but the production of hemotoxin is greatly reduced, indicating that the basic substances from which the hemotoxin is derived are likewise adsorbed, at least in part, by filtration. The fact that the addition of insoluble compounds also removes hemotoxin from bouillon seems to confirm the part played by adsorption in the process of filtration.

Effect of Centrifugation and Shaking on the Hemotoxin.

If the hemotoxin is contained in or on the bacterial cells, concentration of the cells by centrifugation should increase the hemolytic titer. A tube of hemolytic bouillon was centrifuged at high speed until the supernatant liquid was clear (about 20 minutes). Another tube of bouillon fitted with a rubber stopper was placed on a wheel revolving about 30 times a minute and the bouillon was kept in constant agitation for 20 minutes. The top half of the liquid in the centrifuged tube was removed with a pipette; part of this was used for plating on blood agar in order to determine the number of cocci present and part used for hemolytic tests. The lower half was shaken in order to get an even distribution of the sedimented streptococci and was tested in a similar manner. As a control on the presence of streptococci some of the same bouillon was treated with the pulverized filter material for 20 minutes and centrifuged for 3 minutes. The results are shown in Table II.

The counts shown in this table are of necessity inaccurate, since we have no means of knowing whether one streptococcus or a chain of several members produced the colonies. They are, however, relative and give a fair ground for comparison. The hemotoxin has evidently disappeared from the supernatant fluid, although there are some organisms present. Even though the streptococci have been concentrated to twice the number per cubic centimeter the hemolytic titer is slightly less than that of the bouillon in the control tube which had not been centrifuged. The process of shaking also reduced the titer somewhat, although the bacterial count remained the same as in the

control tube. The culture treated with kieselguhr contains enough streptococci to show some hemolysis, provided the hemolytic substance is an integral part of the cell. If a hemolytic test is set up in a hollow culture slide and the process of hemolysis studied through the microscope it soon becomes evident that hemotoxin must be free in the medium and unassociated with the bacterial cells. Red blood corpuscles are seen to assume gradually a moth-eaten appearance and finally to disintegrate, although during the whole process no streptococci have been near them. In such a test as that shown in Table II there are present 100 erythrocytes to each streptococcus. Since these organisms are non-motile it is difficult to believe that each blood cell has come in direct contact with a streptococcus. Microscopic study gives convincing evidence that such is not the case. The results of these experiments seem to indicate that the hemotoxin is not associated with the bacterial cells and that there is a mechanical factor responsible for loss of titer during centrifugation.

TABLE II.

Treatment.	0.5 cc.	0.25 cc.	0.12 cc.	0.06 cc.	0.03 cc.	0.015 cc.	No. of colonies per cc.
Untreated	++	++	++	+	−	−	1,000,000
Centrifuged, top	−	−	−	−	−	−	50,000
" bottom	++	++	+	+	−	−	∞
Shaken	++	++	+	−	−	−	1,000,000
Kieselguhr	−	−	−	−	−	−	100,000

Relation of Hydrogen Ion Concentration to the Hemotoxin.

Certain concentrations of acid and alkali are suitable reagents for causing the laking of erythrocytes. The possibility that hemolysis may be due to this cause cannot be ignored. The presence in bouillon of carbohydrates from which acid might be produced tends, however, to decrease rather than increase the amount of hemotoxin. The only apparent effect of the initial hydrogen ion reaction of the medium was to inhibit growth when the reaction was strongly acid or alkaline. Hemotoxin was obtained from bouillon having a pH value at the time of testing for hemolysis of 7.2 to 5.3. Media containing pieces of marble gave maximum hemotoxin production. Hemolytic bouillon

diluted with an equal volume of isotonic sodium carbonate solution gave results identical with those obtained by diluting to the same extent with sodium chloride solution. From these results it seems impossible that the hemolytic action can be due to the presence of an acid radical.

Relation of the Constituents of the Medium to Hemotoxin Production.

The ideal method of attacking this phase of the problem would be to withdraw one substance after another from the culture medium and test each substance alone and also the remainder of the material as to its ability to support hemotoxin production. This is not possible in all cases on account of the complexity of the constituents of

TABLE III.

Bouillon.	Growth.	0.5 cc.	0.25 cc.	0.12 cc.	0.06 cc.	0.03 cc.	0.015 cc.
Undiluted.....	Good.	++	++	++	++	++	+
Diluted to 2 volumes.....	"	++	++	++	++	++	+
" " 4 "	"	++	++	++	++	++	+
" " 8 "	"	++	++	++	++	+	+
" " 16 "	Slight.	++	++	++	+	−	−
" " 32 "	"	++	++	+	+	−	−
" " 64 "	"	++	+	+	−	−	−
Horse serum + salt solution.....	None.	−	−	−	−	−	−

the bouillon. We must constantly bear in mind the necessity of removing any bactericidal reagent from the medium and also of retaining the tonicity of the preparations at a point such that hemolysis of red blood cells will not follow from this cause.

It seemed probable that on occasions solutions would be added to the medium and it would be inconvenient and not expedient to evaporate the excess. The effect of diluting the bouillon with physiological salt solution was therefore investigated. As usual, 3 drops of horse serum were added to each tube after the dilutions were made.

From Table III it is evident that bouillon can be diluted to four times its volume before any effect on hemotoxin production is observed. Beyond this point the hemotoxin production is in inverse ratio to the dilution of the medium. In no case has the addition

of reagents approached four times the volume of the bouillon so any decrease in hemolytic titer is not due to the factor of dilution.

Relation of Phosphorus to Hemotoxin Production.—In Table I it was shown that the addition of calcium chloride to bouillon caused the formation of a precipitate. Upon analysis this proved to consist mainly of calcium phosphate, although other substances were brought down either mechanically or by adsorption. Phosphoproteins are not precipitated by this reagent. It was found, however, that streptococci during growth liberated phosphorus, presumably from the protein radicals. In order to obtain a phosphorus-free medium the following procedure was adopted. 0.6 cc. of 2.5 per cent calcium chloride solution was added to 5 cc. of bouillon and boiled. The precipitate was removed by centrifugation. The supernatant liquid

TABLE IV.

Bouillon.	Growth.	0.25 cc.	0.12 cc.	0.06 cc.	0.03 cc.	0.015 cc.
Bouillon B_1 with calcium chloride.....	Good.	++	++	++	++	+
" B_2 " " "	"	++	++	++	++	+
" B_3 " " "	"	−	−	−	−	−
" B_3 + ammonium monobasic phosphate.....	"	++	+	+	−	−
Bouillon B_3 + phosphoric acid.....	"	++	++	+	−	−
" B_3 + potassium acid phosphate....	"	++	−	−	−	−

was enriched with 3 drops of normal horse serum, inoculated with streptococci, and incubated for 24 hours. Then the process was repeated until the medium was free from phosphorus. Two or three inoculations were generally necessary. Such a phosphorus-free bouillon reinforced by 3 drops of normal horse serum supported abundant growth of streptococci but allowed production of no hemotoxin.

The addition of known phosphorus compounds to a phosphorus-free bouillon was tried. In Table IV is a tabulation of a series of such experiments. Bouillon B_1 was heated with calcium chloride once; Bouillon B_2 was heated with calcium chloride, the precipitate removed, inoculated with streptococci, incubated, and precipitated a second time. No. B_3 was carried through the process of precipitation for a third time. When chemically tested this bouillon was shown to be free from phosphorus. The amounts of phosphorus compounds

added were 0.5 cc. of 2 per cent ammonium monobasic phosphate, 0.5 cc. of 2 per cent potassium acid phosphate, and 0.5 cc. of 1 per cent phosphoric acid. These solutions are approximately isotonic for red cells as they gave no hemolysis when added to a standard cell suspension.

The results of these experiments indicate that as long as the phosphoproteins are present in the medium the maximum amount of hemotoxin is produced. The addition of phosphorus compounds restores in part but not completely the hemotoxin-producing properties of the medium. The non-production of hemotoxin in phosphorus-free bouillon is not due to the effect of the lack of buffer action, as the reaction was unchanged by the growth of the streptococci and presented a range within which hemotoxin has been produced repeatedly.

Relation of Other Chemical Factors to Hemotoxin Production.—The preceding experiment and certain others suggested the possibility that there might be present in the medium two factors or chemical essentials, one of which is able to support growth but not hemolysin production, the other supplies the necessary substances for the elaboration of hemotoxin. Experiments were instituted to separate the chemical substances contained in infusion bouillon. Metaproteins, proteoses, and peptone were isolated from the bouillon according to the methods outlined by Hawk (1916). These substances were made up to the approximate volume of the bouillon from which they were taken by the addition of physiological salt solution, and the standard amount of normal horse serum was added. Fair growth of streptococci was invariably obtained but no hemotoxin was produced. Even the mixture of all these substances in the original proportion failed to support the production of hemotoxin. It seems evident that a variety of substances may support growth but not the production of hemotoxin.

Two reagents have been used for the fractionating of bouillon, calcium chloride and ether. When calcium chloride is added to broth a heavy precipitate is formed. This precipitate can be divided by ether extraction into a waxy substance, which is perhaps cerebrin, and calcium phosphate. Neither the whole precipitate nor either of the fractions, when washed free of bouillon, supported hemotoxin production. Bouillon after removal of the calcium precipitate suffers

no loss of its hemotoxin-producing qualities. Extraction of this fraction with ether does not affect the production of hemotoxin. When the whole bouillon is extracted with ether and then precipitated with calcium chloride the fractions containing the water-soluble matter are the only ones which give hemolysis. These preparations were brought as nearly as possible to the original volume and to them was added the usual amount of serum.

If the calcium precipitate previously referred to is returned to the bouillon no effect on hemotoxin production is observed. The substance separated from the bouillon by ether extraction, whether obtained from the whole bouillon or from the calcium precipitate, when returned to bouillon considerably reduces the yield of hemotoxin. Pure cholesterol when added to the bouillon does not influence hemotoxin production. Lecithin, however, has a pronounced inhibitory action. Thus the substance which is separated from the bouillon by means of ether acts in a manner similar to lecithin. The substances in bouillon which are soluble or insoluble in ether are not essential to the production of hemotoxin of the streptococcus.

Relation of Serum to Hemotoxin Production.—When bouillon prepared with Liebig's beef extract instead of meat infusion is planted with streptococci, fair growth is obtained but no hemotoxin is produced. By using this medium as a base, opportunity is offered for determining whether the source of the hemotoxin is in the infusion bouillon or in the blood serum. To one series of tubes containing 5 cc. of Liebig's extract bouillon graduated amounts of infusion bouillon were added; to the other the same amounts of serum were added. The results of these tests are shown in Table V.

From this table it can be seen that the infusion bouillon of itself adds but little to the production of hemotoxin, whereas serum increases the production in the ratio of its presence in the medium. There is, perhaps, the requisite amount of the growth factor in either Liebig's or infusion bouillon. The basic substances for hemotoxin production are present in only a slight degree in infusion bouillon but in considerable quantity in serum. It is evident that there are certain quantitative relations between the growth factor and the hemotoxin-producing factors to be observed in the production of the maximum amount of hemotoxin. With infusion bouillon 0.05 cc., or 3 drops, of

serum are sufficient for maximum hemotoxin production, whereas with Liebig's extract bouillon 2 cc. of serum are necessary to obtain the same result. The growth factor in infusion bouillon may be diluted to four volumes as shown in Table III and still produce the maximum amount of hemotoxin. Horse serum diluted with physiological salt solution will support little if any growth.

The isolation of the hemotoxin-producing substance from serum is as difficult as from bouillon. Serum was diluted to twice its volume with salt solution and heated in boiling water until the coagulable proteins were well separated. This preparation was centrifuged until the supernatant fluid, or serum water as we have designated it, was perfectly clear. When this serum water which amounted to 5

TABLE V.

Amount of infusion bouillon + 5 cc. of Liebig's extract bouillon.	Hemolysis produced.					Amount of horse serum + 5 cc. of Liebig's extract bouillon.	Hemolysis produced.				
	0.25 cc.	0.12 cc.	0.06 cc.	0.03 cc.	0.015 cc.		0.25 cc.	0.12 cc.	0.06 cc.	0.03 cc.	0.015 cc.
cc.						*cc.*					
0.05	+	−	−	−	−	0.05	−	−	−	−	−
0.2	++	+	−	−	−	0.2	+	−	−	−	−
0.6	++	+	−	−	−	0.6	++	++	+	+	−
1.0	++	+	−	−	−	1.0	++	++	++	+	−
2.0	++	+	−	−	−	2.0	++	++	++	++	++
2 cc. of infusion + 5 cc. of salt solution.	++	++	++	++	−	2 cc. of horse serum + 5 cc. of salt solution.	−	−	−	−	−

to 10 per cent of the original serum was made up to volume and added to bouillon, hemotoxin was produced in maximum quantity. Calcium chloride solution, when added to serum water, causes a precipitate, but the hemotoxin-producing factor is but slightly diminished in the supernatant fluid. The coagulable protein and the calcium precipitate of serum water do not support hemotoxin production and growth occurs to only a slight degree.

Hemotoxin-Producing Substance in Various Organs.—The occurrence of the hemotoxin-producing substance in different organs was investigated. Water extracts of brain, liver, and kidney were prepared by infusing the macerated tissues in an equal weight of water. The meat and precipitate were removed by centrifugation and the clear

extract was sterilized by boiling for 5 minutes. These preparations were added to Liebig's extract bouillon in amounts of 0.6 cc., a quantity in the case of serum sufficient to produce a fair amount of hemotoxin. The brain extract induced little or no hemotoxin production due, evidently, to the presence of a lipoid in the medium. The liver extract produced an unusual amount of growth but no hemotoxin. This result we considered as due to the selective action of the streptococcus for the glycogen in the liver extract. The kidney extract, however, when added to Liebig's bouillon produced hemotoxin equal in amount to that elaborated in the presence of serum or serum water. These results are similar to those obtained by Kligler (1919) in his study of the growth-producing substances in tissue.

The outstanding feature of these substances is their susceptibility to alkalinity and high temperatures. Kidney infusion was distributed in 5 cc. amounts into four test-tubes. To the first tube 1 cc. of normal potassium hydroxide was added; it was boiled for 5 minutes and then exactly neutralized with normal hydrochloric acid. To the second tube were added 1 cc. of normal potassium hydroxide and 1 cc. of normal hydrochloric acid and the whole was boiled at the same time as the first. To the third tube were added 2 cc. of sodium chloride solution and the entire contents were boiled as a control when the other tubes were boiled. The fourth tube was autoclaved for 30 minutes at 15 pounds pressure. The contents of the first tube when added to Liebig's extract bouillon in any amount failed to support hemotoxin production. The contents of the second and third tubes induced hemotoxin production equal to that obtained with serum. The autoclaved infusion acted in a manner comparable to that of infusion bouillon as shown in Table V.

At first one is tempted to designate the hemotoxin-producing substance by the term vitamine, as did Kligler (1919). It is doubtful whether the word as used by the physiological chemists can be justified in this application in bacteriology. The distinction between the growth factor and the factor producing hemotoxin is marked. A variety of substances may supply the needs for metabolism. Proteoses, peptone, probably some of the amino-acids, and sugars furnish the essentials for growth. The substance essential for hemotoxin production does not support the growth of the organisms. We believe that

hemotoxin is a compound synthesized from at least two basic substances; one of these is a phosphorus compound, the other a substance of unknown composition. The latter is present in fresh tissue and body fluids, is water-soluble, and is susceptible to certain destructive processes. The hemotoxin compound is easily destroyed by mechanical and physical methods and acts as an organic compound and not as an enzyme.

CONCLUSION.

The hemotoxin of streptococcus is a labile substance affected by centrifugation or shaking. It is adsorbed by various organic and inorganic substances. Hemotoxin is produced within a wide range of hydrogen ion concentrations. It is neither in nor on the bacterial cell but is free in the culture medium. It is probably not an enzyme. There are at least two substances which are essential to the medium for the elaboration of hemotoxin, one of which is phosphorus; the other is a substance of unknown composition. The unknown component is present in small quantities in unfiltered muscle infusion, but is more abundantly supplied by blood serum and kidney infusion. This substance is not an albumin, globulin, primary or secondary proteose, metaprotein, or peptone of the medium or enriching fluid. It is water-soluble, is destroyed by boiling in alkaline solution and by prolonged heating, and is removed to a considerable extent by passage through a diatomaceous filter.

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DIVISIONS OF THE SO CALLED FLEXNER GROUP OF DYSENTERY BACILLI.*

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(Received for publication, June 21, 1920.)

INTRODUCTION.

There is considerable confusion in regard to the existence of subdivisions of the mannitol-fermenting group of dysentery bacilli. Numerous attempts have been made to establish subdivisions on the basis of their biological and serological characteristics (1–8). My conclusions are derived from the fermentation and agglutination reactions of 77 cultures isolated from cases of clinical dysentery in children, 12 cultures from cases of dysentery in the American Expeditionary Forces, and stock cultures of the 5 English types of Flexner bacilli.[1]

By the Flexner group I refer to Gram-negative non-motile bacilli, isolated from the stools of cases of clinical dysentery and agglutinated by the patient's serum. They produce a small amount of indole, do not liquefy gelatin, fail to ferment lactose, and produce acid and no gas in dextrose and mannitol. These are the only constant cultural characteristics. It would seem advisable to apply one name, *Bacillus dysenteriæ* Flexner, to this whole group. These mannitol-fermenting dysentery bacilli have been subdivided in two ways, first by fermentation tests with maltose, saccharose, dulcitol, and rhamnose, and secondly by agglutination tests with the sera of immunized rabbits.

* Read before the 7th annual meeting of the American Association of Immunologists, New York, N. Y., April 1, 1920.

[1] For the technique employed and a description of the cases of dysentery in children see Davison, W. C., Bacillary dysentery in children, *Bull. Johns Hopkins Hosp.*, 1920, xxxi, 225.

Divisions Based on Carbohydrate Fermentation.

In this country it has been customary to refer to the Flexner (9), Harris, Hiss-Russell (10), and Strong (11) divisions on the basis of differences in the fermentation of maltose and saccharose. In my series, by utilizing the differences of fermentation of dulcitol and rhamnose, three additional divisions were found in a few cases (Table I).

TABLE I.

Types of Fermentation of Flexner Dysentery Bacilli.

Fermentation type.	Name.	Motility.	Fermentation of test substances.									Per cent of cases.
			Indole.	Gelatin.	Lactose.	Dextrose.	Mannitol.	Dulcitol.	Rhamnose.	Maltose.	Saccharose.	
1	Hiss-Russell.....	0	+	0	0	+	+	0	0	0	0	7
2	Flexner, English Y...,	0	+	0	0	+	+	0	0	+6	0	41
3	Strong.....	0	+	0	0	+	+	0	0	0	+13	14
4	Harris, English V, X..	0	+	0	0	+	+	0	0	+	+15	24
5	English Z.....	0	+	0	0	+	+	0	+12	+4	+12	7
6	*B. alkalescens*.....	0	+	0	0	+	+	+5	0	+	0	3
7		0	+	0	0	+	+	0	+	0	+6	3

If late fermentation occurred, the day of its appearance is designated by the number placed after the + sign. Otherwise + designates acid (no gas) production on the 1st day and indole production on the 7th day. 0 denotes no fermentation (incubation continued 21 days).

Fermentation Type 7 was also isolated from a diarrhea case which was not clinically dysentery.

Other workers using still different carbohydrates have reported additional divisions (6, 12–14). In fact, if fermentation is accepted as a criterion for differentiation, still further members will probably be added in the future as new carbohydrates are used for fermentation tests, and even more confusion will result.

It has been demonstrated (7, 15) that dysentery cultures may lose or gain ability to ferment maltose, saccharose, and also dulcitol (4) and that distinctions made on this basis are inconstant and unreliable. In the same stool I have isolated cultures that fermented maltose and others that failed to do so (Case 53, Table II). In another

TABLE II.

Discrepancies between Fermentation and Agglutination Divisions of Flexner Dysentery Bacilli in Children.

Case No. (Culture No.).	Agglutination type of stool culture.					Summary.	
	Type sera.					Fermentation type.	Agglutination type.
	V	W	X	Y	Z		
53*† (L156), 6* (69, 70, 73)	0	+125	0	±200	0	1	W, Y
6* (72, 74)	±200	0	0	0	0	1	V
39*(L61), 40* (L56, L57, L58)	0	0	0	±200	0	2	Y
39* (L51), 40* (L59), 46* (L82), 53*† (L154), 80* (L11), 10* (161, 174, 175A)	0	0	0	0	0	2	0
46* (L83, L84), 52 (L166, L167), 53*† (L155), 54 (L159)	0	+250	+250	±500	0	2	W, X, Y
80* (L9)	±125	±250	0	±300	0	2	V, W, Y
80* (L10, L12), 81 (L30, L32, L33), 8*† (107, 113, 114, 115, 116), 10* (156, 159, 162, 164, 186, 157)	0	±250	0	±250	0	2	W, Y
9 (153, 154)	+1,250	0	0	0	±500	2	V, Z
Y stock.	+125	0	+250	+625	±100	2	V, X, Y, Z
58 (L174, L175)	0	0	0	0	0	3	0
77 (L186, L187)	0	0	±1,250	+200	±1,250	3	X, Y, Z
5 (67)	+500	0	±200	0	±500	3	V, X, Z
V stock.	+1,250	0	0	+250	0	4	V, Y
X "	0	0	+250	0	0	4	X
41* (L65)	0	0	0	0	0	4	0
41* (L66), 45* (L75, L77), 63* (L114)	0	0	+500	0	+250	4	X, Z
41* (L67)	0	0	0	0	±200	4	Z
41* (L68, L69), 13*† (262, 268)	±200	0	+200	0	+250	4	V, X, Z
45* (L76)	0	+100	+250	±200	0	4	W, X, Y
63* (L115, L116), 78 (L188)	0	0	+500	+200	+150	4	X, Y, Z
79*† (L2, L5), 13*† (261, 263, 266, 270, 271, 274)	±1,000	0	0	0	±250	4	V, Z
13*† (260)	+1,250	0	0	±200	±250	4	V, Y, Z
13*† (267), 79*† (L4)	+500	0	0	0	±500	5	V, Z
Z stock.	0	0	+625	0	+625	5	X, Z
8*† (108)	0	0	0	0	0	6	0
69 (L111, L112), 39 (152)	0	0	0	0	0	7	0

Cases marked with an asterisk (*) had more than one serological type and those marked with a dagger (†) had more than one fermentation type of Flexner dysentery bacillus in the stools. Culture numbers preceded by L, *i.e.* L156, were from cases in Baltimore, the others were from Birmingham, Alabama. + and ± indicate complete and partial agglutination; the numbers following these signs refer to the dilutions (end-point) of the serum. Case 69 was a diarrhea which was not clinically dysentery.

dysentery specimen I have found organisms that differed in dulcitol fermentation (Case 8, Table II). In other stools I have isolated dysentery bacilli that differed in rhamnose fermentation (Cases 13 and 79, Table II).

It is, of course, possible that these represent mixed infections, but this is impossible to prove, inasmuch as the fermentation differences in maltose, saccharose, dulcitol, and rhamnose, as will be pointed out later, do not parallel the serological findings.

Divisions Based on Agglutination with Monovalent Rabbit Sera.

Murray (1) in England has discarded the fermentation divisions of the Flexner group and has found that thirty-four cultures isolated from cases of dysentery in different parts of the world fell into five divisions on the basis of their agglutination reactions with monovalent rabbit sera. These he designates as the V, W, X, Y, and Z divisions of the Flexner group.

As Murray emphasizes, these divisions are not definite but probably indicate that one or more antigens predominate in a given strain. With the English cultures and sera, by means of Dreyer's (16, 17) technique with formolized cultures, I have found that although each serum usually agglutinates its own organism to higher titer than other types (Table III), yet the V serum will also agglutinate the Y culture, the X serum will agglutinate Y and Z, the Y serum will agglutinate the V culture, the Z serum will agglutinate the Y culture, while the W serum, which is of low titer, appears to be specific. Murray states that absorption tests do not make these serological divisions more distinct. It may perhaps be found that agglutination with the sera of animals other than the rabbit may give more clear-cut results, as has been demonstrated (18) with sheep serum for streptococci.

With the cultures which I have isolated there is even more cross-agglutination (Tables II and IV). Some cultures that are agglutinated to end-titer by V serum react to quarter titer with Z. W, X, and Y sera frequently agglutinate the same cultures. With other cultures further combinations were found.

Among 62 cultures from children, of which many reacted with more than one serum (Table II), there were 21 positive agglutination

reactions with V sera, 28 with W, 21 with X, 38 with Y, and 28 with Z, while 15 cultures failed to agglutinate with any of these sera. It is probable that at least 2 of these 15 inagglutinable cultures (Nos. 174 and 175, Tables II and IV) represented a type differing from

TABLE III.

Cross-Agglutination of the Five English Type Sera.

Serum.	Culture.	Agglutination titer.					
		1:100	1:125	1:250	1:625	1:1,250	1:2,500
V	V	+	+	+	+	+	0
	W	0					
	X	0					
	Y	+	+	0			
	Z	0					
W	V	0					
	W	+	+	+	0		
	X	0					
	Y	0					
	Z	0					
X	V	0					
	W	0					
	X	+	+	+	0		
	Y	+	+	+	0		
	Z	+	+	+	+	0	
Y	V	+	+	+	0		
	W	0					
	X	0					
	Y	+	+	+	+	0	
	Z	0					
Z	V	0					
	W	0					
	X	0					
	Y	+	0				
	Z	+	+	+	+	0	

Murray's five, for the patient's serum (Case 58, Table IV) had no agglutinins for the V, W, X, Y, and Z stock cultures. Further artificial cultivation might have rendered the others agglutinable, but the destruction of the laboratory and cultures by fire prevented it.

TABLE IV.

Correlation of Patient's Serum Reactions and Types of Stool Organisms.

Case No.	Length of time after onset.	Patient's serum reactions. Type cultures. V	W	X	Y	Z	No. of culture from stool.	Agglutination type of stool culture. Type sera. V	W	X	Y	Z	Summary. Type of patient's serum reaction.	Agglutination type of stool culture.	Fermentation type of stool culture.
	days														
39	200	0	0	0	±50	0	61	0	0	0	±200	0	Y	Y	2
							51	0	0	0	0	0		0	2
40	205	±200	±200	0	±200	0	56, 57, 58	0	0	0	±200	0	V, W, Y	Y	2
							59	0	0	0	0	0		0	2
46	21	+250	+1,000	+100	+250	±20	83, 84	0	+100	+250	+500	0	V, W, X, Y, Z	W, X, Y	2
							82	0	0	0	0	0		0	2
54	14	+100	0	0	+100	0	159	0	+250	±200	±500	0	V, Y	W, X, Y	2
80	19	+20	±20	0	+200	±20	9	±125	±250	0	±300	0	V, W, Y, Z	V, W, Y	2
	38	+100	±50	0	±200	±20	10, 12	0	±250	0	±250	0		W, Y	2
							11	0	0	0	0	0		0	2
58	6	0	0	0	0	0	174, 175	0	0	0	0	0	0	0	3
77	26	±50	0	0	±50	0	186	0	0	±1,250	+200	±1,250	V, Y	X, Y, Z	3
	76	+20	0	0	0	0	187						V		

41	184	0	±20	0	0	0	66	0	0	+500	0	+250	W	X, Z	4
							68, 69	±200	0	+250	0	+500		V, X, Z	4
							67	0	0	0	0	±150		Z	4
							65	0	0	0	0	0		0	4
63	68	0	0	+20	0	0	115, 116	0	0	+500	+200	+150	X	X, Y, Z	4
							114	0	0	+500	0	±150		X, Z	4
78	13	±20	±20	0	+20	0	188	0	0	+1,250	+500	+500	V, W, Y	X, Y, Z	4
79	17	+200	0	0	+100	+20	2, 5	±1,000	0	0	0	±250	V, Y, Z	V, Z	4
	21	+100	0	0	+100	+50	4	±1,000	0	0	0	±250		V, Z	5
69		0	0	0	0	0	111, 112	0	0	0	0	0	0	0	7

+ and ± indicate complete and partial agglutination; the numbers following these signs refer to the dilutions (end-point) of the serum. 0 indicates no agglutination with the patient's sera at 1:20, and with the diagnostic type sera at 1:150.

In other words, although a distinct division was not obtained, agglutination reactions with the five English type sera and these mannitol-fermenting dysentery bacilli lead to the assumption that different antigens exist and that there are more than five which must be recognized.

Discrepancies between Fermentative and Serological Divisions.

There is no correlation between the divisions by fermentation tests and the serological results. Organisms of the same fermentation reactions were agglutinated by different sera. As an example (Table II), among the group that ferment maltose and saccharose, usually called the Flexner-Harris division (Fermentation Type 4), some cultures were agglutinated only by the Z serum, some by the X, Y, and Z, some by the V and Z, some by the X and Z, some by the W, X, and Y, and still others by the V, X, and Z sera, etc. Conversely, organisms agglutinated by the same sera may have different fermentation reactions. It was also noted that among organisms from the same stool with the same fermentation reactions, some were agglutinated by one or more of the sera while others were inagglutinable.

With the English stock cultures (Tables I and III) the same lack of correlation exists; for instance, the Y and X cultures differ in saccharose fermentation, yet both are agglutinated by the X serum.

Levine (3) has laid emphasis on the fact that the Z culture is the only one to ferment rhamnose, yet it is agglutinated by the X as well as the Z serum. Two rhamnose fermenters isolated from dysentery cases were agglutinated by both V and Z sera. Three rhamnose fermenters, isolated from cases of diarrhea which were not clinical dysentery, were not agglutinated by Z or any of the other sera. Furthermore, the Z serum agglutinated many strains that did not ferment rhamnose. From one stool (Case 79, Table IV) one organism that fermented rhamnose and two that failed to ferment it were agglutinated by V and Z sera.

Dulcitol-fermenting dysentery bacilli from one case were agglutinated by the Y serum and those from another were inagglutinable. This division has been named *Bacillus alkalescens* by Andrewes (2) who regards it as non-pathogenic.

Correlation of Agglutination Reactions of the Patient's Serum and Fermentation and Serological Types of His Stool Organisms.

A partial correspondence exists between the serological type of the organisms isolated from the patient's stool and the agglutination reactions of his serum.

In all except one case (Table IV), if the patient's serum agglutinated any of the five stock antigens, the organisms isolated from the stool reacted with one or more of the sera of the same types. In many cases, however, the stool organisms were not agglutinated by all the types for which the patient had agglutinins. As an example, one patient's serum (Case 40, Table IV) had agglutinins for Types V, W, and Y, while the dysentery bacillus isolated from his stool reacted with only the Type Y serum.[2]

The reverse of this was also true; namely, that the patient's serum would not have agglutinins for all the types which were represented by his stool organisms; for instance, the organisms isolated from a patient's stool (Case 63, Table IV) were agglutinated by the X, Y, and Z sera, while his serum had agglutinins for only the X antigen. These illustrations indicate an overlapping of the antigenic content of these strains.

In the only instance in which there was no correspondence (Case 41, Table IV), the serum of the patient agglutinated the W culture while the organisms from the stool reacted with the V, X, and Z sera.

In a few cases in which tests were repeated after various intervals, if agglutinins persisted, they were of the same types that had been present in previous tests.

CONCLUSIONS.

From these data it is seen that ill defined divisions of the so called Flexner group exist. The divisions do not appear to be sufficiently distinct to warrant the use of separate names. To avoid confusion all mannitol-fermenting dysentery bacilli should be called *Bacillus*

[2] It might be argued that had more colonies been studied in the stool culture, Types V and W might have been found; but this would not explain the instances in which the patient's serum did not have agglutinins for all the types found in his stool.

dysenteriæ Flexner and the subdivision noted. There are two methods for this division, one by the fermentation of carbohydrates, the other by agglutination with monovalent rabbit sera. These do not coincide and one or the other and not both must be adopted. Inasmuch as Murray studied organisms from widely distributed sources, it would seem preferable to adopt his serological classification and to add to it the types that fail to be agglutinated by his V, W, X, Y, and Z sera, as this method is simpler and more rapid. The results of the agglutination reactions of the patient's serum may be expressed in the same terms as the serological typing of the organism from his stool. Fermentation is less constant and gives rise to more divisions than there are carbohydrates.

TABLE V.

Agglutinin Content of Polyvalent Therapeutic Serum.

Serum.	Culture.	Agglutination titer.							
		1:100	1:125	1:250	1:625	1:1,250	1:2,500	1:6,250	1:12,500
Polyvalent therapeutic serum.	V	+	+	+	+	+	+	0	
	W	+	+	+	+	+	+	+	0
	X	+	+	0					
	Y	+	+	+	+	+	+	+	0
	Z	+	+	+	+	+	0		

The serological reactions of these type sera, as Murray points out, show cross-agglutination to a greater or less extent, but they indicate that there are five antigens, V, W, X, Y, and Z and probably others, one or more of which predominate in a given strain. Polyvalent diagnostic and therapeutic sera are practically worthless unless they include antibodies for the more common of these types.

The diagnostic importance of recognizing that there are five or more antigens in this group is seen from the fact that the sera of some patients react with one, others with another, and that unless several antigens are used, some positive tests may be missed.

The therapeutic importance is emphasized by the fact that probably the best polyvalent therapeutic serum at present available has a very low titer for the X antigen, although that type was found in many of these cases (Table V).

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BIOLOGICAL STUDY OF THE HEMOPHILIC BACILLI.

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In 1892 Pfeiffer[1] described a small Gram-negative bacillus which he associated with the disease influenza. Ultimately this bacillus was quite widely considered as the cause of influenza. The bacillus of Pfeiffer, or *B. influenzæ*, as it was eventually named, is a very small, non-motile, non-sporulating, faintly staining organism with rounded ends. It is irregular in form with a tendency to show bipolar staining. Coccoid forms are also frequently seen, and occasionally small chains of bacilli occur. Pfeiffer recovered the bacillus by smearing pus from bronchial secretions over serum agar, but subcultures failed to grow. He finally discovered that it was the hemoglobin in the pus which enabled the bacillus to grow. Thus this organism came to be known as a hemophilic bacillus. Since Pfeiffer's discovery, various Gram-negative hemophilic bacilli have been described, such as the pseudoinfluenza bacillus, Jochmann's bacillus, Muller's "trachoma bacillus," the Koch-Weeks bacillus, etc. The more recent studies of Wollstein[2] would seem to indicate that while there are minor morphological and cultural differences between these hemophilic bacilli, the distinctions are so slight that the various hemophilic bacilli should be considered identical with *B. influenzæ* or, at most, as varieties of the same species.

The epidemic of influenza in 1918 called to attention the lack of knowledge concerning the biology and epidemiology of the hemophilic bacilli despite the large amount of work done in connection with influenza. In a study of the occurrence of *B. influenzæ* in normal mouths, Pritchett and Stillman[3] described a hemophilic bacillus strikingly similar to, but distinguished from *B. influenzæ* by its ability to hemolyze blood. The colony of this organism cannot be differentiated from that of *B. influenzæ* on oleate agar or chocolate medium, and morphologically the differences are so slight that they cannot be relied upon. As a rule, the so called Bacillus X is slightly larger and coarser than *B. influenzæ* in stained films. The easiest method of differentiation is by growth on blood agar, upon which Bacillus X shows varying degrees of hemolysis. The majority of the strains of this organism actively hemolyze the surface of a blood agar plate and also hemolyze blood broth. An occasional strain is encountered, however,

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[2] Wollstein, M., *J. Exp. Med.*, 1915, xxii, 445.

[3] Pritchett, I. W., and Stillman, E. G., *J. Exp. Med.*, 1919, xxix, 259.

whose hemolytic powers are not well developed. If good growth is obtained in plain broth enriched with 2 per cent blood extract, hemolysis may be demonstrated by the use of a 5 per cent solution of washed rabbit blood corpuscles. 1.5 cc. of an 18 hour broth culture added to 0.5 cc. of the washed blood cells and placed in a water bath at 37.5°C. for 1 hour usually cause complete hemolysis. Jordan[4] in 1919 called attention to the fact that certain strains of *B. influenzæ* produced indole. Wadsworth and Wheeler,[5] in their work with *B. influenzæ*, note the production of gas, and also the fermentation of monosaccharides by some strains. Rivers[6] reported that certain strains of *B. influenzæ* produced indole and amylase and could reduce nitrates to nitrites.

In 1906 Bordet and Gengou[7] succeeded in cultivating a small, ovoid, Gram-negative bacillus which they had observed in the sputum of children suffering from pertussis. This bacillus, although very similar morphologically to *B. influenzæ*, is less pleomorphic, slightly larger, and generally appears more ovoid. After frequent subcultures *B. pertussis* grows on ordinary media without the presence of hemoglobin. It grows much more slowly during the first 24 hours of incubation than *B. influenzæ*. On blood agar a fine film of growth is barely visible at the end of 24 hours, while at the end of 48 hours a heavy grayish growth has developed which is very different from the appearance of *B. influenzæ* grown under the same conditions. Ferry and Noble[8] have stated that there is an apparent close relation between *B. pertussis* and *B. bronchisepticus*, although the latter grows luxuriantly on ordinary media and is motile. The bacillus of rabbit septicemia, while not hemophilic, presents a striking morphological likeness to *B. influenzæ*. Because of the morphological resemblance of these various bacilli to *B. influenzæ* a few strains are included for comparison in the present study of the hemophilic bacilli.

As the hemophilic bacilli are delicate organisms which do not grow readily on artificial media special attention must be paid to minute details of technique. This fact is well exemplified by the difficulty with which *Bacillus influenzæ* was cultivated before the use of special media such as oleate agar and chocolate agar and probably in large part accounts for our lack of knowledge of the biology of this delicate organism. Freshly prepared medium adjusted to the optimum hydrogen ion concentration, pH 7.3 to 7.5, is essential for growth.

[4] Jordan, E. O., *J. Am. Med. Assn.*, 1919, lxxii, 1542.

[5] Wadsworth and Wheeler, in Park, W. H., and Williams, A. W., Pathogenic microörganisms, Philadelphia and New York, 7th edition, 1920, 457.

[6] Rivers, T. M., *Bull. Johns Hopkins Hosp.*, 1920, xxxi, 50.

[7] Bordet, J., and Gengou, O., *Ann. Inst. Pasteur*, 1906, xx, 731.

[8] Ferry, N. S., and Noble, A., *J. Bact.*, 1918, iii, 193.

EXPERIMENTAL.

In the present paper are reported the data obtained during an investigation of the hemophilic bacilli recovered from the throats and sputum of patients suffering from acute influenza and lobar pneumonia, and from the throats and saliva of healthy individuals. The study includes the facts obtained concerning (1) final hydrogen ion concentration, (2) sugar fermentation, (3) indole production, (4) nitrate reduction, and (5) gas production. In addition to the strictly hemophilic organisms, *Bacillus influenzæ* and the so called Bacillus X, described by Pritchett and Stillman, a few strains of *Bacillus pertussis*, the bacillus of rabbit septicemia, and *Bacillus bronchisepticus* have been included for comparative study.

Upon isolation the majority of the strains of *Bacillus influenzæ* and all the strains of Bacillus X were plated on dextrose agar to which no hemoglobin had been added. In no instance was growth obtained in the hemoglobin-free medium. After prolonged artificial cultivation, in some instances over 2 years, all these strains were again plated on ascitic dextrose agar without hemoglobin. They invariably failed to grow on media which lacked hemoglobin. All media used in this study were enriched by the addition of 4 per cent defibrinated rabbit blood or 2 per cent blood extract. The latter was substituted for defibrinated rabbit blood in the case of the hemolytic Bacillus X, since the hemolysis produced by this organism might mask certain reactions. In many instances also in the work with the non-hemolytic hemophilic bacilli (*Bacillus influenzæ*) when defibrinated blood might interfere with the determination of a reaction, blood extract was used to enrich the media. The extract was made as advised by Wollstein.[9] Defibrinated rabbit blood was boiled for 2 minutes. The clot was finely broken and centrifuged. The resulting extract was added to the media in such a proportion as to give about 2 per cent enrichment. Since Winchell and Stillman[10] found that the optimum hydrogen ion concentration for growth of *Bacillus influenzæ* is between pH 7.3 and 7.5, all media used in the present study had an initial reaction of about pH 7.4 unless otherwise stated.

[9] Wollstein, M., *J. Exp. Med.*, 1919, xxx, 555.

[10] Winchell, A. I., and Stillman, E. G., *J. Exp. Med.*, 1919, xxx, 497.

Non-Hemolytic Hemophilic Bacilli (Bacillus influenzæ).

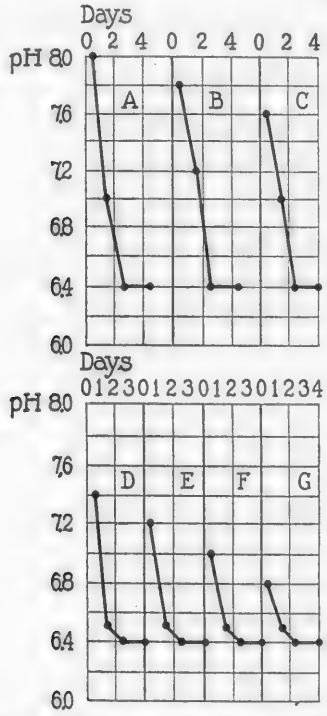
Hydrogen Ion Concentration.—Since the final hydrogen ion concentration reached by growth of an organism in a given medium is a biological constant of differential value, this reaction was determined in the study of the hemophilic bacilli. The colorimetric method, with phenol red and brom cresol purple as indicators, was used to determine the hydrogen ion concentration, and the readings were confirmed by the electrometric method in a number of experiments.

In the usual dextrose meat infusion broth containing 0.5 per cent sodium chloride, *Bacillus influenzæ* attains a final acid reaction of about pH 6.2. If 0.2 per cent sodium phosphate is substituted for the 0.5 per cent sodium chloride in this medium, as is customary for routine purposes in these laboratories, the buffer value is so great that the changes in reaction are insignificant. Since *Bacillus influenzæ* evidently produces relatively small amounts of acid it is desirable in determining the final hydrogen ion concentration to use the medium containing less buffer. Consequently, throughout this work broth containing 0.5 per cent sodium chloride has been used.

In order to determine whether the initial hydrogen ion concentration of the medium had any effect on the final reaction, separate portions of dextrose broth, adjusted to varying hydrogen ion concentrations from pH 8 to 6.8, were inoculated with the same culture of *Bacillus influenzæ*. Text-fig. 1 shows the initial hydrogen ion concentration of the broth when inoculated and the hydrogen ion concentration of the cultures after 20, 44, and 70 hours incubation. From this it is seen that the initial reaction bears no relation to the final hydrogen ion concentration, which is pH 6.4 in each instance.

The relation of oxygen supply to growth was next tested by inoculating a series of 100 cc. Erlenmeyer flasks containing 25 cc. of dextrose broth and a set of large test-tubes containing a similar amount of broth. The initial hydrogen ion concentration of the media was pH 7.3. The test-tube cultures were incubated in an upright position. Colorimetric readings of the hydrogen ion concentrations of the cultures were made after 1, 3, 7, and 14 days incubation. After 24 hours incubation the flask cultures had attained a pH of 6.4, while the test-tube cultures did not reach this end-point until the 7th to 10th day.

It was noted that the macroscopic appearance of the cultures is not a criterion of the hydrogen ion concentration. Cultures which are very turbid and apparently have grown well, when tested may be found not to have reached their lower limit of acid production.



TEXT-FIG. 1. Effect of different initial hydrogen ion concentrations on final hydrogen ion concentrations of *B. influenzæ*.

The final pH was determined on a large series of cultures of *Bacillus influenzæ*. It was found to lie between pH 6 and 6.4. The length of incubation necessary before the different strains, and even the same strain, reach their final hydrogen ion concentration varies. Some strains reach the final reaction at the end of 24 hours; others, at times, do not reach pH 7 even after 14 days incubation in large slanted test-tubes. This variability of growth of *Bacillus influenzæ* has been

encountered throughout the present study. In working with this organism experiments giving negative results must be repeated, since the results may be due merely to insufficient growth.

Sugar Fermentation.—Since all the cultures of *Bacillus influenzæ* ultimately reached a final hydrogen ion concentration of at least pH 6.4 in dextrose broth, a reaction sufficiently acid to be detected by the Andrade indicator, the ability of these organisms to ferment different sugars was tested. It was found that sugar-free broth could not be used as nutrient substrate even after enrichment with blood extract and the addition of the test substance, for *Bacillus influenzæ* did not readily produce acid in this medium. Since meat infusion broth contains varying amounts of muscle sugar which might possibly modify fermentation reactions, Dunham's peptone solution was employed as a basis for the sugars, which were added in 1 per cent concentration. The peptone solution does not contain sufficient reducing sugar to give a positive test with Benedict's reagent. In Dunham's peptone solution enriched with 2 per cent blood extract *Bacillus influenzæ* grows luxuriantly. All culture tubes were incubated in a slanted position so as to expose to the air as large a surface of the medium as possible, since it has been shown that acid production is more rapid under these conditions.

The results of the sugar fermentation are given in Table I. It is seen that almost all strains of *Bacillus influenzæ* produce acid in the monosaccharides, dextrose and galactose. Acid production is less marked and more irregular with levulose. Some strains of *Bacillus influenzæ* fermented the polysaccharides, maltose and saccharose, and to a less extent dextrin. No strains could attack mannitol or lactose. A number of strains were tested against inulin, but the results were so consistently negative that this test substance was subsequently discarded. It is evident that the sugar reactions are irregular. This irregularity is especially noticeable if the same strain is repeatedly inoculated in the same sugar. Although good growth was apparently present, a culture which had been repeatedly positive in dextrose, for instance, at times failed to produce sufficient acid to cause the Andrade indicator to change color. The same factors which sometimes prevented cultures from reaching their final hydrogen ion concentration of pH 6.4 in dextrose broth apparently were acting here.

TABLE I.

Source, Sugar Fermentation, Indole Formation, Nitrate Reduction, and Gas Production of the Strains of Non-Hemolytic Hemophilic Bacilli (B. influenzæ).

Source of strain.	Total No. of strains.	Strains fermenting sugars.																Strains producing indole.		Strains reducing nitrates.		Strains producing gas.		Strains producing hemolysis.	
		Dextrose.		Galactose.		Levulose.		Maltose.		Saccharose.		Dextrin.		Mannitol.		Lactose.									
		No.	Per cent.	No.	Per cent.	No.	Per cent.	No.	Per cent.	No.	Per cent.	No.	Per cent.	No.	Per cent.	No.	Per cent.	No.	Per cent.	No.	Per cent.	No.	Per cent.	No.	Per cent.
Acute influenza	21	20	95	20	95	13	61.9	4	19	2	9	3	14	0	0	0	0	14	66	21	100	1	4	0	0
Convalescents from influenza	11	11	100	9	82	10	91	0	0	0	0	1	9	0	0	0	0	8	72	11	100	0		0	0
Lobar pneumonia	18	18	100	18	100	8	44	2	11	1	5	1	5	0	0	0	0	12	66	18	100	1	5	0	0
Normal individuals during winter of 1918–19	33	32	97	28	85	24	72	5	15	6	18	4	12	0	0	0	0	18	54	31	94	2	6	0	0
Normal individuals during Sept., 1919	11	10	91	9	82	8	72	5	45	5	45	5	45	0	0	0	0	5	45	11	100	5	45	0	0
Normal individuals during Feb., 1920	25	25	100	24	96	24	96	15	60	16	64	14	56	0	0	0	0	6	24	25	100	7	28	0	0
Total	119	116	97	108	90	87	73	31	26	30	25	28	23	0	0	0	0	63	53	117	98	16	13	0	0

Hydrogen ion determinations were made on a number of strains grown in media containing different sugars. The strains of *Bacillus influenzæ* which showed fermentation with the Andrade indicator had a final hydrogen ion concentration of pH 6 to 6.4 which corresponded to that obtained in dextrose broth when no Andrade indicator was present. An interesting result was the reaction of *Bacillus influenzæ* in lactose. In this sugar there was a definite increase in alkalinity, as illustrated by Table II. If plain peptone solution, peptone solution plus dextrose, and peptone solution plus lactose are inoculated with the same culture of *Bacillus influenzæ* and incubated for 10 days the dextrose culture becomes acid, but the culture in plain peptone and that containing lactose become alkaline. Bacillus X produces a similar increase in alkalinity in lactose media.

TABLE II.

Final Hydrogen Ion Concentration of the Non-Hemolytic Hemophilic Bacilli in Peptone Solution, Peptone Solution Plus Lactose, and Peptone Solution Plus Dextrose.

Solution.	pH
Peptone	8.4+
" + 1 per cent lactose	8.4+
" + 1 " " dextrose	6.0

In the course of the work on sugar fermentation Bronfenbrenner's double indicator "CR" was tested.[11] This indicator is composed of equal parts of a 0.5 per cent aqueous solution of China blue and a 1 per cent alcoholic solution of rosolic acid. China blue appears blue or bluish green in the presence of acid, and colorless in the presence of alkali. Rosolic acid, which is colorless in an acid medium, becomes pink in an alkaline medium.

Dunham's peptone solution containing 1 per cent sugar concentrations and enriched with 2 per cent blood extract was used. 1 per cent CR indicator was substituted for the Andrade indicator previously used. Only two sugars, dextrose and lactose, were tested. Representative strains of the Gram-negative bacilli under observation were used in determining the value of CR as an indicator of acid and alkali production in the presence of these two sugars.

[11] Bronfenbrenner, J., *J. Med. Research*, 1918–19, xxxix, 25.

The hemolytic and non-hemolytic strains of the strictly hemophilic bacilli produced acid in the dextrose CR medium and alkali in the lactose CR medium as indicated by the definite color changes after 48 hours incubation. The intensity of the reaction increases with prolonged incubation. The bacillus of rabbit septicemia also produced acid in the presence of dextrose and alkali in the presence of lactose. The strains of *Bacillus pertussis* and *Bacillus bronchisepticus* produced alkali in both the dextrose and lactose media. These results correspond exactly with the results obtained with similar sugar media in which Andrade indicator replaced CR, as will be seen by referring to Tables I, III, and IV.

CR, in the concentration used, does not appear to be bactericidal for any of the strains tested in the fluid medium described, for good growth was obtained in each instance and the color changes which occurred were striking. In poured oleate hemoglobin agar plates to which CR and the desired sugar had been added in 1 per cent concentration, striking color changes did not occur. In the concentration used in solid medium CR did not appear to be of value as a differential indicator.

Indole Production.—Jordan has called attention to the production of indole by *Bacillus influenzæ*. The indole production by cultures included in this study was tested by Ehrlich's para-dimethylaminobenzaldehyde method. Of the 119 strains of *Bacillus influenzæ* studied, 63, or 53 per cent, produced indole. It was found that indole was present at times after only 18 hours incubation at 37°C. and was produced for as long a period as 3 weeks. Indole was produced quite regularly in plain blood broth cultures, but slightly more positive reactions were obtained if Dunham's peptone solution enriched with blood extract was used. This may be due to the fact that the defibrinated blood masked delicate reactions. The same irregularity of reaction that was noted in the fermentation of sugar by *Bacillus influenzæ* was observed in the production of indole; occasionally an indole-producing strain, which apparently had grown luxuriantly, failed to produce indole. Hence the necessity of repeated tests before a culture may be definitely classified as a non-indole producer. A possible relation exists between indole production and sugar fermentation. Only one indole-producing strain fermented the polysaccharides.

Nitrate Reduction.—Of the 119 strains of *Bacillus influenzæ* studied, 117 were able to reduce nitrates to nitrites. This reduction as a rule occurred after 24 hours incubation. Like other reactions with these delicate organisms, at times negative results were obtained without apparent reason. In a few instances a culture which had given a positive nitrite reaction after 24 hours incubation failed to give it after 10 days incubation.

Gas Production.—The ability of the hemophilic bacilli to produce gas was first tested by making stab cultures in 1 per cent dextrose agar to which a small amount of blood extract had been added. Under these partial anaerobic conditions luxurious growth was obtained with all strains. Of the 119 non-hemolytic strains, sixteen, or 13 per cent, were found to produce gas. The gas appeared usually after 48 to 72 hours incubation, but with several strains did not appear until later. The time of incubation necessary before the appearance of gas varied on different occasions when the same strain was used. The amount of gas produced was never very great.

Stab cultures were made with blood extract dextrose medium containing agar in concentrations of 1, 1.5, and 2 per cent. 1 per cent agar seemed to be the most favorable concentration for the demonstration of gas. Gas production was demonstrated also in shake cultures of dextrose blood extract agar. With the exception of one strain, the non-hemolytic gas-producing organisms did not produce gas in Smith fermentation tubes when meat infusion broth or Dunham's peptone solution containing dextrose and blood extract was used. It appears, therefore, that a solid medium is more suitable than a fluid medium for the production of gas by these non-hemolytic strains of hemophilic bacilli.

Hemolytic Hemophilic Bacilli (Bacillus X).

The hemolytic hemophilic bacilli reach a final hydrogen ion concentration which varies from pH 6.4 to 5.8. Most strains ferment dextrose, maltose, and saccharose readily and quite regularly, and utilize galactose, levulose, and dextrin less easily and more irregularly. The same irregularities and difficulties of growth have been encountered with this organism as with the non-hemolytic type, although it is not quite so variable. Of the twenty-nine hemolytic strains studied only

three produced indole after repeated attempts. All the strains reduced nitrates to nitrites. Only four strains, or 13 per cent, showed the production of gas in dextrose blood extract agar. With these organisms the gas appeared usually after 24 to 48 hours of incubation. With only one of the strains could gas production be demonstrated in a Smith tube with meat infusion broth containing dextrose and blood extract. Stab and shake cultures were made in a similar manner as described above in connection with the non-hemolytic organisms.

TABLE III.

Sugar Fermentation, Indole Formation, Nitrate Reduction, and Gas Production of the Strains of Hemolytic Hemophilic Bacilli (Bacillus X) Isolated from Normal Individuals during the Winter of 1919–20.

Total No. of strains.	Strains fermenting sugars.																		Strains producing indole.		Strains reducing nitrates.		Strains producing gas.		Strains producing hemolysis.	
	Dextrose.		Galactose.		Levulose.		Maltose.		Saccharose.		Dextrin.		Mannitol.		Lactose.		Inulin.									
	No.	Per cent.	No.	Per cent.	No.	Per cent.	No.	Per cent.	No.	Per cent.	No.	Per cent.	No.	Per cent.	No.	Per cent.	No.	Per cent.	No.	Per cent.	No.	Per cent.	No.	Per cent.	No.	Per cent.
29	29	100	3	10	9	31	24	82	23	79	15	51	0	0	0	0	0	0	3	10	29	100	4	13	29	100

Relation between Indole Formation and Gas Production.

An apparent relation between indole formation and gas production can be observed. All the non-hemolytic gas-producing strains are non-indole producers and comprise strains that ferment mono- as well as polysaccharides. The hemolytic gas-producing strains, with one exception, produce indole. These hemolytic gas-producing strains do not ferment sugars so readily as the other organisms in this group.

Comparative Study of Bacillus pertussis, the Bacillus of Rabbit Septicemia, and Bacillus bronchisepticus.

Table IV shows the results of a comparative study of *Bacillus pertussis*, the bacillus of rabbit septicemia, and *Bacillus bronchisepticus* in connection with the hemophilic bacilli.

TABLE IV.

Sugar Fermentation, Indole Formation, Nitrate Reduction, and Gas Production of Strains of B. pertussis, the Rabbit Septicemia Bacillus, and B. bronchisepticus.

Bacillus.	Total No. of strains.	Strains fermenting sugars.																		Strains producing indole.		Strains reducing nitrates.		Strains producing gas.		Strains producing hemolysis.	
		Dextrose.		Galactose.		Levulose.		Maltose.		Saccharose.		Dextrin.		Mannitol.		Lactose.		Inulin.									
		No.	Per cent.	No.	Per cent.	No.	Per cent.	No.	Per cent.	No.	Per cent.	No.	Per cent.	No.	Per cent.	No.	Per cent.	No.	Per cent.	No.	Per cent.	No.	Per cent.	No.	Per cent.	No.	Per cent.
B. pertussis	4	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Rabbit septicemia bacillus	4	4	100	4	100	4	100	0	0	4	100	0	0	4	100	0	0	0	0	4	100	2	50	0	0	0	0
B. bronchisepticus	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

Bacillus pertussis.—The four strains of *Bacillus pertussis* studied were stock cultures which had been under artificial cultivation for a considerable length of time. They had a final hydrogen ion concentration of pH 8 to 8.6 in dextrose broth and failed to produce acid in any of the sugars tested. Neither did they produce indole or reduce nitrates to nitrites. In dextrose agar stab cultures there was only a slight growth below the surface and no gas was produced.

Bacillus of Rabbit Septicemia.—The four strains of the bacillus of rabbit septicemia had a final hydrogen ion concentration of pH 6 and produced acid in dextrose, galactose, levulose, saccharose, and mannitol. They did not produce acid in maltose, lactose, dextrin, or inulin. These organisms produced indole slowly, and two strains, or 50 per cent, reduced nitrates to nitrites. In dextrose agar stab cultures there was good growth in the stab, but no gas was produced.

Bacillus bronchisepticus.—The two strains of *Bacillus bronchisepticus* studied had a final hydrogen ion concentration of pH 9.2 in dextrose broth and failed to produce acid in any of the sugars tested. They did not produce indole or reduce nitrates to nitrites. In dextrose agar stab cultures there was only a slight growth below the surface and no gas was produced.

DISCUSSION.

The small Gram-negative hemophilic bacilli which have gradually come to be considered as belonging to one group of organisms and to which the name *Bacillus influenzæ* has been given, appear in the light of the present study to be rather a group of closely allied bacilli which have demonstrable biological differences. The bacillus which Pfeiffer first described and associated with clinical influenza is now questioned as being the etiological factor in the spread of this disease. However, the percentage of cases in which the bacillus of Pfeiffer has been recovered is great enough to indicate that this organism may be at least a secondary invader. Since the first description of this hemophilic bacillus in 1892 by Pfeiffer, little has been added to our knowledge of its biological characteristics.

In this study we have found that the hemophilic bacilli observed divide themselves naturally into two large groups according to their

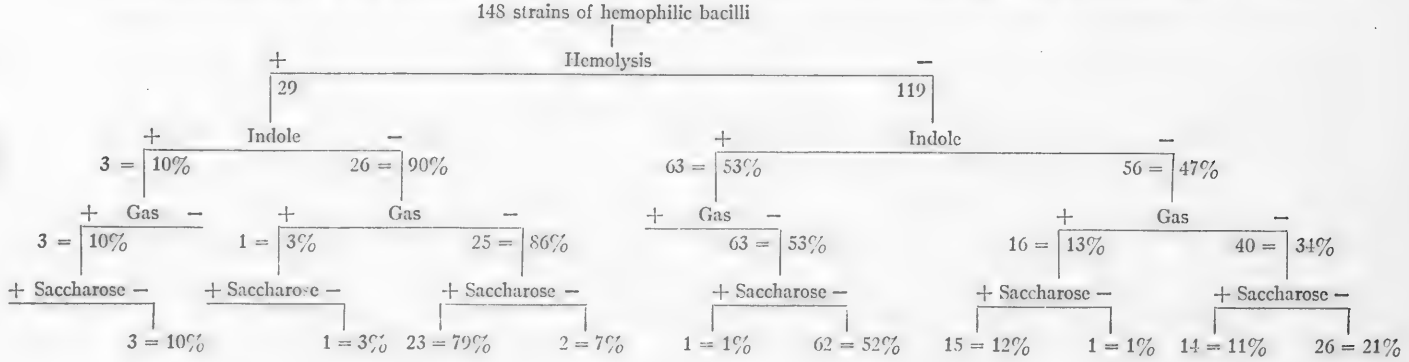
ability to hemolyze whole blood. The hemolytic group comprises the organisms originally described as Bacillus X by Pritchett and Stillman, and occurs in normal mouths. Many of these hemolytic bacilli have no doubt been confused with the non-hemolytic variety due to the almost universal use of chocolate medium. On oleate agar the colonies are so similar that they cannot be distinguished, and morphological differences are so slight as not to be reliable. Organisms of the hemolytic type (Bacillus X) do not live so long in culture media as those of the non-hemolytic type. They are best preserved at a low temperature. A few strains have been found to live from 2 to 3 weeks if kept in blood broth in the ice chest, but in order to be successfully preserved in stock cultures they must be transplanted every 6 or 7 days. At room temperature Bacillus X survives about 5 days, while at 37.5°C. it remains viable about 10 days. The non-hemolytic group (*Bacillus influenzæ*), on the other hand, remains viable for a month or more at room temperature in blood broth.

The hemolysin produced by the hemolytic type is quite stable, retaining its activity after being kept on ice for 6 weeks to several months. It can be demonstrated in a young broth culture after 2 hours incubation at 37°C. It is non-filterable and is destroyed by heating for $\frac{1}{2}$ hour at 56°C. Different strains vary, however, in their ability to produce hemolysis. The hemolytic bacilli are non-pathogenic for rabbits, guinea pigs, and mice.

Both the non-hemolytic and hemolytic groups of hemophilic bacilli attain a final hydrogen ion concentration of approximately pH 6.4, although the hemolytic group may reach pH 5.8. Both produce acid in dextrose, but in both groups only certain strains ferment saccharose. The greater ability of the hemolytic organisms to ferment sugars may be a basis for further differentiation.

A tentative classification, graphically illustrated below, defines a small subgroup of the hemolytic group formed by the strains which produce indole and gas but do not ferment saccharose. These strains appear to ferment sugars less readily and require further study to determine whether the indole-producing strains are also gas producers. The greater number of hemolytic strains, however, do not produce indole or gas, but ferment saccharose.

Tentative Classification of the Hemophilic Bacilli Based on Hemolysis, Indole and Gas Production, and the Fermentation of Saccharose.



The non-hemolytic organisms are subdivided into two fairly even groups comprising indole-producing and non-indole-producing strains. None of the indole producers forms gas, in contrast with the hemolytic group. With one exception, none of the non-hemolytic indole-producing strains ferments saccharose. A large majority of the non-indole-producing organisms of the non-hemolytic type do not form gas and do not ferment saccharose. With a single exception, all the indole-negative strains which form gas also ferment saccharose.

One of the most striking features of this classification may be best illustrated by Table V, which represents a comparison of three factors differentiating the hemolytic and non-hemolytic groups of the hemophilic bacilli. Here it is seen that the majority of the strains of the hemolytic type do not produce indole or gas, but ferment saccharose,

TABLE V.

Three Differential Factors of the Hemolytic and Non-Hemolytic Groups of the Hemophilic Bacilli.

Differential factors.	Hemolytic group.		Non-hemolytic group.	
	Positive.	Negative.	Positive.	Negative.
	per cent	*per cent*	*per cent*	*per cent*
Saccharose	79	21	25	75
Gas	13	86	13	87
Indole	10	90	53	47

while the reverse is true of the non-hemolytic type; that is, the majority of the non-hemolytic organisms do not produce gas but also do not ferment saccharose. It will be noted that the non-indole and indole-producing strains of the non-hemolytic type fall into almost even groups.

The classification made in this study is merely a tentative one. Undoubtedly when the technique of the reactions is more nearly perfected and a larger number of hemophilic bacilli has been studied, the group differentiations will be more striking and regular.

Although the number of strains of *Bacillus influenzæ* employed is too small to warrant any definite conclusions, it would seem that the non-hemolytic bacilli isolated from individuals suffering with and recovering from respiratory infections and those isolated from normal mouths during the epidemic period differ biologically in certain re-

spects from the strains recovered from normal individuals during the winter of 1919–20. This point is illustrated by Table VI. It is seen that the group of non-hemolytic hemophilic bacilli recovered from normal mouths during the winter of 1919–20 shows a higher percentage of strains which ferment the polysaccharides, maltose, saccharose, and dextrin, and more strains which produce gas, but fewer indole-producing strains.

TABLE VI.

Comparison of the Strains of Non-Hemolytic Hemophilic Bacilli Recovered from Respiratory Infections and Normal Mouths during Epidemic Period of 1918 and Strains Recovered from Normal Individuals during the Winter of 1919–20.

Source of strain.	Total No. of strains.	Strains fermenting sugars.												Strains producing indole.		Strains producing gas.	
		Dextrose.		Galactose.		Levulose.		Maltose.		Saccharose.		Dextrin.					
		No.	Per cent.	No.	Per cent.	No.	Per cent.	No	Per cent.	No.	Per cent.	No.	Per cent.	No.	Per cent.	No.	Per cent.
Respiratory diseases and normal mouths during epidemic of 1918, and respiratory diseases during 1919–20.	83	81	97	75	90	55	66	11	13	9	10	9	10	52	62	4	4
Normal mouths during winter of 1919–20.	36	35	97	33	91	32	88	20	55	21	58	19	52	11	30	12	33

The Gram-negative bacilli which are not hemophilic and which have been studied because of their morphological similarity can be easily differentiated from the hemolytic and non-hemolytic hemophilic bacilli of the influenza type. The bacillus of rabbit septicemia shows a striking similarity to members of the non-hemolytic hemophilic group in the limiting hydrogen ion concentration, indole production, and nitrate reduction. On the contrary, *Bacillus pertussis* and *Bacillus bronchisepticus*, while resembling each other in certain reactions, do not simulate the strictly hemophilic group. These organisms have a markedly alkaline final hydrogen ion concentration, and do not produce indole or reduce nitrates.

CONCLUSIONS.

1. The hemophilic bacilli can be divided into two large groups according to the ability of certain strains to produce hemolysis.

2. Both the hemolytic and the non-hemolytic groups may be further subdivided according to the ability of some strains to produce indole, to form gas, and to ferment certain carbohydrates.

3. The hemophilic bacilli of both the hemolytic and the non-hemolytic varieties when grown in meat infusion broth containing 1 per cent of dextrose reach a final hydrogen ion concentration of about pH 6.4. In addition, practically all the strains possess the power to reduce nitrates to nitrites.

We wish to express our thanks to Dr. N. S. Ferry for a culture of *Bacillus bronchisepticus*, to Dr. Martha Wollstein for cultures of *Bacillus pertussis* and the bacillus of rabbit septicemia, to Dr. Anna Williams for cultures of *Bacillus pertussis*, to Dr. F. S. Jones for a culture of *Bacillus bronchisepticus*, and to the American Museum of Natural History for a culture of *Bacillus pertussis*.

FURTHER STUDIES ON THE ETIOLOGICAL RÔLE OF VIBRIO FETUS.*

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Among the twenty-six cases of abortion associated with *Vibrio fetus*,[1, 2] of which one also contained *Bacillus abortus*, there was none involving heifers. All were second or later pregnancies. All but three were purchased cows. More recently three cases of abortion in native heifers have been found associated with *Vibrio fetus*. These cases are of sufficient importance to be given in detail.

No. 433.—Native heifer; aborted October 24, 1919. Records state that she had been bred February 22, 1919. Male fetus, 28 inches long; color black and white. Coat of hair still lacking. The stomach fluids contained a few particles of meconium only. The contents of the large intestine were normal. The lungs were free from air. There were some small hemorrhages in the auriculo-ventricular valves. Besides a general fullness of blood in the organs there were no marked abnormalities in the fetus.

Cultures were made as follows: From the contents of the fourth stomach, which showed spirals in films, pure cultures of spirilla were obtained. Films from small and large intestines were negative, but cultures developed spirilla from contents of the colon and rectum. Cultures from the lungs and spleen also contained spirilla only. Those from the liver and kidneys remained sterile.

One guinea pig was inoculated with the contents of the fourth stomach, another with meconium, and a third with lung tissue. Killed after 7 weeks, none showed any signs of disease due to *B. abortus*, and the spleen cultures remained sterile.

A portion of the placenta representing the unoccupied horn, which had passed out, was cut from the still adherent and retained remainder. It was covered with shavings and without any odors of decomposition. During the washing to

* The tests relating to the milk were made by Miss Marion L. Orcutt.

[1] Smith, T., *J. Exp. Med.*, 1918, xxviii, 701.

[2] Smith, T., *J. Exp. Med.*, 1919, xxx, 313.

remove adherent bedding, yellowish, cheesy particles were carried off by the wash water from the cotyledons.

The chorion varies from a smooth, translucent, slightly injected membrane to one opaque, thickened, and leathery. The opacity is due to an infiltration which is in the form of slightly elevated plaques not removable by gentle scraping. In other places the infiltration is discrete, in the form of whitish opacities, ½ to 1 mm. in diameter. There are also scattering minute tufts of adventitious villi, completely cheesy. Edema of the subjacent tissue varies in thickness from place to place. The cotyledons are in part normal, in part diseased. Some of the latter are yellowish, pultaceous throughout. The still normal cotyledons contain, usually on the margin, necrotic, yellowish villi. More rarely such villi are scattered through the cotyledon.

Many films were examined without showing any bacilli of the type of *B. abortus*. There were, however, occasional larger rods and vibrios. A few were closely wound, the rest flatter, as is usual with *V. fetus*.

Sections of fixed and hardened material from various regions of the placenta show necrosis of villi, loss of surface epithelium with infiltration of the underlying tissue with leucocytes in certain areas. Where epithelium is present, no bacteria are found in them, as is the case when *B. abortus* is present. The endothelium of the capillaries has proliferated in places and it partly or nearly fills the lumina. Bacteria resembling vibrios are detected within these cells and in groups among necrotic villi. Inoculation of two guinea pigs with scrapings from the diseased placenta was negative as regards *B. abortus*.

Milk collected 3 days after the expulsion of the fetus, centrifuged according to the method previously described,[3] and injected into three guinea pigs also yielded negative results as regards *B. abortus*. Agglutinins for *B. abortus* were absent from the milk at this time.

The blood agglutinations of this heifer are significant in that they indicate subsequent infection with *B. abortus* during the second pregnancy. 2 days before abortion, October 22, 1919, the titer limit was 1: 20, 20 days after abortion a trifle above 1: 40. Nearly 6 months later samples taken 4 days apart were 1: 640.

No. 438.—Date of breeding uncertain. Aborted November 9, 1919. The placenta was retained. Fetus, female, measures 17½ inches and weighs about 5 pounds. No subcutaneous edema or serous effusions. Autolytic changes of organs without any distinctly putrefactive odors present. The fourth stomach contains a thick mucoid pinkish fluid and films show spirilla. Films of contents of colon and rectum do not show any bacteria. One ventral lobe of the lungs shows under pleura fine, branching, grayish yellow lines made up of masses of spherical crystals.

Cultures from contents of the fourth stomach contain only spirilla. The same is true of cultures of the colon contents. One culture from the rectum contains

[3] Smillie, E. W., Little, R. B., and Florence, L., *J. Exp. Med.*, 1919, xxx, 341.

cocci, the other only spirilla. Cultures made with bits of spleen, liver, and lungs develop only spirilla. One tube from the kidney contains large bacilli, the other only spirilla.

Four guinea pigs, inoculated respectively with a suspension of material from a uterine swab, with fourth stomach contents, meconium, and lung tissue, were negative as to *B. abortus* when killed and cultured after 6 to 7 weeks.

A sample of milk drawn 9 days after expulsion of the fetus was inoculated into three guinea pigs. *B. abortus* was not isolated from any of them. The titer limit of the agglutinations for *B. abortus* of this heifer 4 days after abortion was 1:40, the milk titer was negative when tested 9 days after. 5 months after abortion the titer limit of the blood was 1:80, of the milk below 1:20.

No. 449.—Aborted during the night of December 1 to 2, 1919. Date of breeding unknown. Female fetus, 16 inches long. Lower jaw and part of upper jaw eaten away by some animal during the night. Some coils of the small intestine protruding from the opening at the umbilicus. General suffusion of tissues with hemoglobin. Autolytic changes indicated by appearance of tissues. Skin readily peeled away from subjacent muscular tissue.

Contents of the fourth stomach turbid. They contain epithelial growths from amnion, swallowed by the fetus. Films from the fourth stomach contents contain numerous spirilla. One culture from the contents contains only spirilla. In the other they are associated with bacilli. Cultures from the liver contain only spirilla. Those from the lungs contain a mixture of rods and cocci.

Only one guinea pig was inoculated with the contents of the fourth stomach. The result was negative as to *B. abortus*.

Milk collected 1 day after expulsion of the fetus was tested on three guinea pigs for *B. abortus* with negative result.

The agglutination titer of this heifer also indicated absence of *B. abortus*. On February 12, 1919, and April 1, it was 1:80. On November 25 it was 1:20. On the date of abortion, December 2, it was 1:40. $4\frac{1}{2}$ months later it was 1:80. The agglutination titer limit of the milk 1 day after abortion was 1:40, $4\frac{1}{2}$ months later it was below 1:20.

Agglutination Tests.

It was stated in an earlier publication[4] that the agglutinability of recently isolated strains of *Vibrio fetus* is low or absent and that it rises under artificial cultivation to a certain maximum. This is not always true, however, as Table I shows. The vibrios of the three aborting cases are titrated against the serum of a rabbit treated repeatedly with cultures of Strain 67. In all cases a high agglutina-

[4] Smith, T., and Taylor, M. S., *J. Exp. Med.*, 1919, xxx, 299.

tion titer is demonstrated, although the strains are still in the early generations. The table also shows a general serological relationship among the strains tested.

The outcome of several tests of the blood serum of the three reported cases is summarized in Table II. Owing to a slight spontaneous agglutination in the controls, the high titers with Vibrio 356 will have to be reduced one or two degrees. The results, while not uniform, give encouragement that agglutination tests may be of use should this type of infectious abortion become more widespread. In

TABLE I.

Agglutination Tests with Serum from a Rabbit Immunized with Cultures of Vibrio 67.

Vibrio No.	No. of transfer.	Serum dilutions.								Control.
		1:20	1:40	1:80	1:160	1:320	1:640	1:1,280	1:2,560	Vibrios in salt solution.
433	11th	C.*	C.	C.	C.	C.	C.	+++	+++	−
438	9th	"	"	"	"	"	"	C.	+++[1]	−
449	5th	"	"	"	"	"	"	"	+++	−
67	125th	"	"	"	"	"	"	"	+++	−

* C. indicates complete agglutination, +++ nearly complete agglutination, ++ marked agglutination, + slight agglutination, ± doubtful, and − no agglutination.

[1] when following a symbol denotes a degree of agglutination between it and the next higher symbol.

only one case (No. 449) was serum antedating the abortion on hand, and this was negative. We have, therefore, no way of knowing at present whether a high titer precedes abortion, as is almost regularly true in the presence of *Bacillus abortus*. By choosing some easily agglutinable strain which has been tested against normal sera it may be possible to detect this type of infection as readily as the other. All of the strains included in the table were tested against the serum of a normal heifer and no clumping was observed in dilutions ranging from 1:20 to 1:1,280.

TABLE II.

Agglutination Tests with Blood Serum of the Three Cases.

Case No.	Date of abortion.	Date of withdrawal of blood.	Vibrio used as antigen.		Date of test.	Serum dilutions.								Controls.	
			Case No.	Transfer.		1:20	1:40	1:80	1:160	1:320	1:640	1:1,280	1:2,560	Vibrios in salt solution.	Normal serum.
433	Oct. 24, 1919	Nov. 13, 1919	433	6th	Dec. 1, 1919	+++[1]	+++[1]	+++[1]	+++[1]	+++	++	−	−	−	−
		" 13, 1919	347	27th	" 1, 1919	+++[1]	+++[1]	+++	++	−	−	−	−	−	−
		Apr. 15, 1920	433	16th	Apr. 19, 1920	C.	C.	+++[1]	+++	+++	++	+	±	±	−
		" 15, 1920	356	32nd	" 19, 1920	+++[1]	+++[1]	+++[1]	+++[1]	+++[1]	+++	+++	+	+	−
438	Nov. 9, 1919	Nov. 13, 1919	438	4th	Dec. 1, 1919	++	+++[1]	+++[1]	+++[1]	+++	++	−	−	−	−
		" 13, 1919	347	27th	" 1, 1919	+++	+++	+++	++	−	−	−	−	−	−
		Apr. 15, 1920	449	16th	Apr. 19, 1920	C.	C.	+++[1]	+++	−	−	−	−	−	−
		" 15, 1920	356	32nd	" 19, 1920	"	+++[1]	+++[1]	+++[1]	+++[1]	+++	+++	++	+	−
449	Dec. 1, 1919	Nov. 25, 1919	449	7th	Jan. 31, 1920	−	−	−	−	−	−	−	−	−	−
		" 25, 1919	347	33rd	" 31, 1920	++	+	−	−	−	−	−	−	−	−
		Dec. 2, 1919	449	5th	" 19, 1920	++[1]	−	−	−	−	−	−	−	−	−
		" 2, 1919	347	33rd	" 31, 1920	+++	++	+	−	−	−	−	−	−	−
		Apr. 15, 1920	449	13th	Apr. 19, 1920	++	−	−	−	−	−	−	−	−	−
		" 15, 1920	356	32nd	" 19, 1920	+++[1]	+++	+++	+++	+++	++[1]	++	+	+	−

GENERAL SUMMARY.

The data bearing on these three cases are quite sufficient to rule out *Bacillus abortus* as the agent. Not only the cultures and guinea pig tests of fetal tissues and contents of the digestive tract, but also the agglutination and guinea pig tests of the milk, were negative. The same is true of the agglutination tests of the blood serum. Only in one case was the placenta obtained in part. The stained films and the sections from various regions showed no abortion bacilli. Guinea pig tests of placental tissue were negative for *Bacillus abortus*. On the other hand, minute organisms resembling vibrios were detected in the cytoplasm of endothelial cells within capillaries in the edematous subchorionic tissue. Subsequently the agglutination titer of the blood serum of one of these cases rose to a level indicating infection with *Bacillus abortus* during the second pregnancy.

The peculiar distribution of abortions due to *Vibrio fetus* among older cows and heifers in this herd, resulting at first in cases among older cows and latterly passing to young stock, may be explained by certain occurrences in the herd itself. It may be assumed that the infection was originally brought in by purchased cows. The young stock is kept segregated from these in a special barn, and when 6 months old it is pastured on outlying farms until returned in an advanced stage of pregnancy. The heifers during the first pregnancy were thus kept away from vibrio carriers until after the first calf was born.

In June and July, 1919, 55 older cows, purchased and native, were placed on the young stock pasture. The three cases of abortion in heifers due to *Vibrio fetus* occurred October 24, November 9, and December 2, 1919. The age and condition of the fetuses accord very well with the assumption that *Vibrio fetus* was introduced among the young stock in June or July of the same year.

The information gathered thus far concerning vibrionic abortion in this herd enables us to formulate a tentative hypothesis subject to modification with increasing knowledge of this type of infectious abortion. The infectious agent was probably introduced by purchased cows in 1917 or earlier. It gained a certain headway up to 1919, then the number of cases declined so that between May, 1919,

and May, 1920, only the above three cases in heifers, and one case of mixed infection with *Bacillus abortus* in an older cow, were detected. During the same period cases due to *Bacillus abortus* continued undiminished. The greater resistance of *Bacillus abortus* manifested in cultures as compared with *Vibrio fetus* is thus reflected in its behavior in nature. The temporary dying out of the infection indicates that natural immunization of a herd to *Vibrio fetus* proceeds quite rapidly. Another outbreak may be expected when the immunity of the herd has declined in the absence of the infecting agent and the latter is reintroduced from without, or it may reappear at any time when a vibrio of higher virulence is brought in.

STUDIES ON EXPERIMENTAL PNEUMONIA.

IX. Production in Monkeys of an Acute Respiratory Disease Resembling Influenza by Inoculation with Bacillus influenzæ.

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The present paper deals with the production in normal monkeys of an acute respiratory disease by inoculation of the mucous membranes of the upper respiratory tract with *Bacillus influenzæ* and of bronchopneumonia by intratracheal injection.[1] These experiments were undertaken in continuation of previous studies[2, 3, 4] on experimental pneumococcus and hemolytic streptococcus pneumonia in monkeys with the purpose of obtaining evidence concerning the relation of *Bacillus influenzæ* to influenza and of determining whether the type of bronchopneumonia that has been ascribed[5, 6, 7] to *Bacillus influenzæ* infection of the lungs in man could be reproduced experimentally in animals.

In general, animal inoculation experiments with *B. influenzæ*, though serving to demonstrate[8,9,10] the pathogenicity of at least some strains of the organism for laboratory animals, have had little actual bearing on the relation of *B. influenzæ* to influenza, since the methods of inoculation have been by the subcutaneous, intraperitoneal, or intravenous routes, and it is not to be expected that a disease

[1] Blake, F. G., and Cecil, R. L., *J. Am. Med. Assn.*, 1920, lxxiv, 170.
[2] Blake, F. G., and Cecil, R. L., *J. Exp. Med.*, 1920, xxxi, 403.
[3] Blake, F. G., and Cecil, R. L., *J. Exp. Med.*, 1920, xxxi, 445.
[4] Blake, F. G., and Cecil, R. L., *J. Exp. Med.*, 1920, xxxii, 401.
[5] Pfeiffer, R., *Z. Hyg. u. Infectionskrankh.*, 1893, xiii, 357.
[6] MacCallum, W. G., *J. Am. Med. Assn.*, 1919, lxxii, 720.
[7] Wolbach, S. B., *Bull. Johns Hopkins Hosp.*, 1919, xxx, 104.
[8] Cohen, *Ann. Inst. Pasteur*, 1909, xxiii, 273.
[9] Wollstein, M., *Am. J. Dis. Child.*, 1911, i, 42; *J. Exp. Med.*, 1915, xxii, 445.
[10] Albert, H., and Kelman, S. R., *J. Infect. Dis.*, 1919, xxv, 443.

which may safely be classified as a respiratory infection could readily be reproduced in animals by introduction of the virus by other than the natural path of infection in man. Attempts have been made, however, to produce influenza by inoculation of the mucous membranes of the respiratory tract with *B. influenzæ*. Pfeiffer[5] inoculated monkeys and other laboratory animals on the mucous membranes of the upper respiratory tract, intratracheally, and by direct injection into the lung, but failed satisfactorily to reproduce influenza or to establish the fact that actual infection took place, though many of the animals suffered from a temporary intoxication and sometimes died. Wollstein and Meltzer[11] produced bronchopneumonic lesions in dogs by massive intrabronchial insufflation of *B. influenzæ*. Wahl, White, and Lyall[12] inoculated the nasal and nasopharyngeal mucous membranes of healthy men with four strains of *B. influenzæ* with negative results. Similar experiments conducted by the United States Public Health Service[13] likewise yielded negative results.

Strain of Bacillus influenzæ Used.

Since it seemed apparent that there was no adequate method available for distinguishing between possible saprophytic and pathogenic varieties of Gram-negative hemophilic bacilli to which the name *Bacillus influenzæ* is at present indiscriminately applied, it seemed desirable to employ a single strain which could be considered with reasonable certainty to be the infecting organism in the case from which it was isolated. Accordingly, the strain of *Bacillus influenzæ*[14] used, designated B, was one originally isolated in pure culture from the pleural exudate withdrawn from a case of empyema following influenzal pneumonia in a child. It conformed to such criteria as we possess for identifying *Bacillus influenzæ* in that it was a small, Gram-negative, non-motile, non-spore-bearing, non-hemolytic bacillus, growing readily in characteristic colonies on suitable hemoglobin-containing media at 37°C., but failing to grow in the absence of hemoglobin or at 20°C. It produced indole. When received it had been grown on artificial media for about 6 weeks and was found to possess practically no virulence for white mice, the 18 hour growth on a whole brown blood agar plate not being lethal on intraperitoneal inoculation.

[11] Wollstein, M., and Meltzer, S. J., *J. Exp. Med.*, 1912, xvi, 126.

[12] Wahl, H. R., White, G. B., and Lyall, H. W., *J. Infect. Dis.*, 1919, xxv, 419.

[13] *Public Health Rep., U. S. P. H.*, 1919, xxxiv, 33.

[14] We are indebted to Dr. T. M. Rivers of the Johns Hopkins Medical School, Baltimore, for the strain of *B. influenzæ* used.

Method of Increasing the Virulence of Bacillus influenzæ.

A preliminary intratracheal test inoculation of a monkey having resulted in no evidence of infection, it was evident that the virulence of the strain of *Bacillus influenzæ* would have to be raised if successful results were to be obtained. This was accomplished by successive animal passages through a series of eleven white mice followed by a series of thirteen monkeys. These animals were inoculated intraperitoneally with broth or 0.85 per cent salt solution suspensions of *Bacillus influenzæ* made from brown blood agar plate cultures. At the beginning of the mouse series the growth from one-half of a plate culture was inoculated. 6 to 8 hours after inoculation the mouse was killed and a plate culture of the peritoneal exudate was made for inoculation of the next mouse on the following day. This procedure was adopted because a few preliminary experiments had shown that the influenza bacillus began to disappear from the peritoneal exudate by 8 hours after inoculation and was entirely gone by 24 hours. With the successive inoculations the organism became progressively more virulent for mice until at the end of the series the growth on one-fourth of a plate killed a mouse in 6 to 8 hours with peritonitis and septicemia.

Following the mouse series the organism was passed by similar methods through thirteen monkeys. The monkeys received large inoculations varying from the growth on four to that on fourteen plates. Cultures for the next succeeding inoculation were made either from peritoneal exudate withdrawn by a sterile capillary pipette 6 to 8 hours after intraperitoneal injection, or from the peritoneal exudate at autopsy in the monkeys that succumbed. Some delay occurred in this series owing to secondary invasion of the peritoneal cavity by *Staphylococcus albus* in some of the monkeys. When this occurred it was necessary to prepare subcultures for the next passage in order to eliminate the staphylococcus. The symptoms produced by the inoculations were those of peritonitis and profound intoxication. In monkeys in which leucocyte counts were made a rapidly developing and very marked leucopenia was found, the counts falling in 3 to 6 hours after inoculation from the normal range of 15,000 to 25,000 down to 2,500 to 10,000 per c. mm., and remaining low until death or recovery took place. The series of passages is shown in Table I.

TABLE I.

Passage of B. influenzæ through Monkeys by Intraperitoneal Inoculation.

Passage monkey No.	Date of inoculation.	Culture of *B. influenzæ*.	Amount inoculated.	Result.	Culture of peritoneal exudate.	
					6–8 hrs. after inoculation.	Autopsy.
	1919					
P 1	July 29	B. 11[1]*	7 plates.	Recovered.	*B. influenzæ.*	
" 2	" 30	" 11.1[1]	4 "	"	" "	
					Staphylococcus albus.	
" 3	Aug. 2	" 11.2[3]	9 "	Aug. 3. Died.	*B. influenzæ.*	*B. influenzæ.*
" 4	" 3	" 11.3[1]	5½ "	" 4. "	" "	" "
						Staphylococcus albus.
" 5	" 6	" 11.4[2]	8 "	" 7. "	" "	*B. influenzæ.*
" 6	" 7	" 11.5[1]	5 "	Recovered.	" "	
" 7	" 9	" 11.6[2]	12 "	Aug. 10. Died.	" "	
" 8	" 12	" 11.7[2]	11 "	" 14. "	" "	*B. influenzæ.*
" 9	" 14	" 11.8[2]	12 "	" 15. Killed.	" "	" "
" 10	" 16	" 11.9[2]	14 "	Recovered.	" "	" "
					Staphylococcus albus.	
" 11	" 19	" 11.10[2]	10 "	"	*B. influenzæ.*	
" 12	" 20	" 11.11[1]	10 "	Aug. 20. Killed.		
" 13a	" 22	" 11.12[2]	10 "	Recovered.		*B. influenzæ.*
					B. influenzæ.	
" 13b	" 25	" 11.12[2]	12 "	Aug. 26. Died.	Diphtheroid bacillus.	
						B. influenzæ.

* B. 11 indicates *B. influenzæ*, Strain B, 11 mouse passages. B. 11.1, etc., indicate Strain B, 11 mouse passages, 1 monkey passage, etc. The exponents indicate the number of cultures removed from the last passage animal.

At the completion of the series 0.1 cc. of a 16 hour blood broth culture of *Bacillus influenzæ* from the peritoneal exudate of Passage Monkey 13b killed a white mouse within 48 hours, 1 cc. in 5 hours. To indicate the rapidity with which this increased virulence was lost by subculture on artificial media, it may be stated that after five transplants in blood broth at 24 hour intervals 1 cc. failed to kill a mouse.

Method.

Two species of monkeys were used, *Cebus capucinus* and *Macacus syrichtus*. All were normal healthy animals as far as could be determined by preliminary observation. No procedures to lower resistance or to injure the respiratory tract were resorted to before inoculation. It is well recognized[15] that the use of monkeys for experimental purposes requires great care in the interpretation of febrile reactions and of variations in the leucocyte count as evidences of infection. To establish the production of disease in the monkey the summation of clinical symptoms, temperature, and leucocyte reactions and the results of bacteriological and pathological examinations are essential. This is particularly true in the case of inoculations with *Bacillus influenzæ*, since it is clearly necessary, in view of the fairly well established toxic effects of influenza bacillus cultures and culture filtrates,[16,17] to show that the results obtained are due to actual infection with *Bacillus influenzæ* and are not merely the result of a temporary intoxication. The methods of study after inoculation, therefore, were the careful observation and recording of clinical symptoms, the taking of morning and evening rectal temperatures, daily total and differential counts of the leucocytes, microscopic examination of stained films of the respiratory tract secretions, and the taking of cultures of the nasal secretion and blood at irregular intervals. Since the disease produced did not prove fatal, most of the animals were killed by a sharp blow on the skull either during the acute stage of the disease or shortly after apparent recovery, and autopsies with bacteriological examination were immediately performed. Whenever

[15] Draper, G., and Hanford, J. M., *J. Exp. Med.*, 1913, xvii, 517.
[16] Parker, J. T., *J. Immunol.*, 1919, iv, 331.
[17] Huntoon, F. M., and Hannum, S., *J. Immunol.*, 1919, iv, 167.

Bacillus influenzæ was recovered in cultures during life or at autopsy it was identified as such by appropriate cultural tests.

Because of the extraordinary rapidity with which *Bacillus influenzæ* may lose its virulence when subcultured on artificial media outside the animal body, a particular effort was made to inoculate the monkeys with cultures as recently removed as possible from the passage monkeys. The material used for inoculation consisted of (1) first or second subculture on brown blood agar plates or in blood broth of *Bacillus influenzæ* recovered from the peritoneal exudate of monkeys of the passage series described above; (2) original or first subculture of *Bacillus influenzæ* recovered from the respiratory tract of a monkey (No. 138) killed during the acute stage of the pneumonia produced by intratracheal injection of the organism; or (3) the peritoneal exudate of monkeys of the passage series dying of *Bacillus influenzæ* peritonitis and septicemia. Comparatively young cultures—10 to 16 hours incubation—were used for inoculation. When plate cultures were employed the bacteria were washed off and suspended in sterile broth so that 1 cc. was equivalent to the growth on one plate. All cultures used for inoculation were tested for purity and identified as *Bacillus influenzæ*. In using peritoneal exudate directly the absence of bacterial contamination was controlled by microscopic examination of stained films before inoculation, and the material was subsequently shown by culture to contain only *Bacillus influenzæ*. The exudate was inoculated at varying intervals within an hour after removal at autopsy, being kept meanwhile at 37°C.

Two methods of inoculation were employed with different purposes in view. In one series of monkeys in order to determine whether *Bacillus influenzæ* would initiate an infection of the upper respiratory tract and, if so, what the characteristics of the disease produced might be, the material to be inoculated was introduced into the nose and mouth either by application to the mucous membranes with a cotton swab previously dipped in the culture material or by instillation with a pipette. In a second series of monkeys the material was introduced into the lower respiratory tract by direct intratracheal injection according to the method previously described.[2] The purpose of this series of experiments was to determine whether pneumonia could be experimentally produced in monkeys with *Bacillus influenzæ*, and, if

so, whether it would present the pathologic characteristics that have been ascribed to pure *Bacillus influenzæ* pneumonia in man by Pfeiffer,[5] MacCallum,[6] Wolbach,[7] and others.

EXPERIMENTAL.

Effect of Inoculating Monkeys on the Mucous Membranes of the Upper Respiratory Tract with Bacillus influenzæ.

Twelve monkeys received inoculations of *Bacillus influenzæ* on the mucous membranes of the nose or nose and mouth (Table II). The amount of culture or peritoneal exudate used in this series of experiments varied from what could be introduced by a single application in each nostril and the mouth of a cotton swab previously dipped in the material to be inoculated, up to 1 cc. of a blood broth culture of *Bacillus influenzæ* instilled into each nostril and 1 cc. into the mouth by a pipette. In every instance there resulted an acute respiratory infection which appeared to be essentially similar to influenza in man.

Clinical Course and Symptoms.—The onset occurred quite suddenly from 3 to 5 hours after inoculation. The first symptom was a variable degree of prostration, often extreme, the animals in many cases lying flat on the floor of the cage, eyes closed and very stuporous, sometimes pressing their hands tightly on the top of the head. This picture was in striking contrast with the onset of pneumococcus or streptococcus pneumonia in monkeys, for in these diseases prostration at onset was never observed, and it was frequently difficult to recognize by inspection that a monkey had become sick except for the accelerated respiration. Symptoms of upper respiratory tract infection soon followed. Frequent sneezing, blinking of the eyes, and rubbing of the nose were the prominent manifestations.

The subsequent course of the disease, though showing some variation in different monkeys, particularly with respect to the development of complications, was in general that of a self-limited infection, the acute stage of which lasted from 3 to 5 days. By the end of 24 hours a variable amount of nasal secretion had appeared, at first mucoid and scanty, later in some instances becoming more profuse and mucopurulent. Stained films and cultures of this secretion in some cases failed to show *Bacillus influenzæ* or at most showed only small num-

TABLE II.

Effect of Inoculating Monkeys on the Mucous Membranes of the Upper Respiratory Tract with B. influenzæ.

Monkey No.	Species.	Weight.	Date of inoculation.	Material inoculated.	Strain of *B. influenzæ* inoculated.	Amount inoculated.		Result.	Complications.	*B. influenzæ* recovered at autopsy.	
						Nose.	Mouth.			U. R. T.	L. R. T.
		gm.	*1919*			*cc.*	*cc.*				
150	*C. capucinus.*	1,350	Aug. 25	10 hr. blood broth culture.	B. 11.11[2]*	2.0	1.0	Acute respiratory disease. Improving on 4th day; killed.		−	+
154	" "	1,740	" 26	Peritoneal exudate of Monkey P 13b.	" 11.13[0]	Swab.	Swab.	Acute respiratory disease. Improving on 4th day; killed.			+
153	" "	950	" 26	" "	" 11.13[0]	1.0		Acute respiratory disease. Killed on 4th day.	Acute sinusitis, left antrum.	−	−
140	" "	1,600	" 26	" "	" 11.13[0]	Swab.	Swab.	Acute respiratory disease. Improving on 4th day; killed.		−	+
148	" "	2,160	" 26	" "	" 11.13[0]	"	"	Acute respiratory disease. Killed on 3rd day.		+	−
142	" "	1,300	" 26	" "	" 11.13[0]	0.5		Acute respiratory disease. Killed on 4th day.	Acute sinusitis, left antrum; bronchopneumonia, R. L.	−	−
139	" "	1,405	" 26	" "	" 11.13[0]	0.5		Acute respiratory disease. Killed on 3rd day.	Acute sinusitis, both antra.	+	−

157	*M. syrichtus.*	5,200	Aug. 26	Peritoneal exudate of Monkey P 135.	B. 11.13[0]	2.0	1.0	Acute respiratory disease. Killed on 4th day.	Acute sinusitis, both antra. Bronchiolitis; bronchopneumonia, L. U., R. U., R. L.	+	+
158	" "	5,675	" 27	15 hr. blood broth culture.	" 11.13[3]	2.0	1.0	Acute respiratory disease. Recovered by 6th day.		No autopsy.	
159	" "	5,430	" 27	" "	" 11.13[3]	2.0	1.0	Acute respiratory disease. Killed on 6th day.	Acute sinusitis, both antra; bronchiolitis; bronchopneumonia, R. U., L. U., L. M., L. L.	+	+
160	*C. capucinus.*	1,500	" 27	" "	" 11.13[3]	1.5	1.0	Acute respiratory disease. Recovered by 6th day.		No autopsy.	
161	" "	1,340	" 27	" "	" 11.13[3]	1.5	1.0	Acute respiratory disease. Recovered by 6th day.		"	"

* This culture was a strain of *B. influenzæ* recovered at autopsy from Monkey 138 (Table III).

R. L., R. U., etc., indicate lobes of the lungs. U. R. T. indicates upper respiratory tract, including the antra; L. R. T., lower respiratory tract.

bers phagocytosed by polymorphonuclear leucocytes; in other cases *Bacillus influenzæ* was present in immense numbers. By 24 to 48 hours after inoculation the infection had spread to the lower respiratory tract, as evidenced by the development of a racking cough which suggested an inflammatory irritation of the trachea and bronchi. This symptom was striking in comparison with the cough of monkeys with pneumococcus pneumonia in which coughing occurred only late in the disease and was comparatively infrequent. That it was due to actual infection of the trachea by *Bacillus influenzæ* was shown by the recovery of the influenza bacillus from the trachea at autopsy, either in pure culture or in association with bacteria that are normally present in the mouths of monkeys.

The febrile reaction generally was not great. Four animals showed no elevation throughout the course of the disease. The character of the temperature curve was inconstant and bore no apparent relation to the severity of the infection, the afebrile animals being as sick as those that showed elevation of temperature. One fairly constant feature was a fall in temperature on the 2nd or 3rd day followed by a secondary rise which appeared to be associated with the development of tracheobronchitis.

The leucocyte counts during the active stage of the disease showed either a definite degree of leucopenia or no significant variation from the normal. The differential counts showed a very brief preliminary polymorphonuclear percentage increase followed by a fall to normal or subnormal level. In two cases a leucocytosis developed on the 2nd and 4th days respectively, autopsies in these instances showing a complicating purulent sinusitis.

Complications.—An acute purulent sinusitis affecting one or both antra developed in five monkeys. At autopsy the antra were found to contain a purulent exudate and the mucous membranes were edematous and congested. Cultures from the antra showed abundant influenza bacilli in three cases, twice in pure culture. In the other two none was recovered, although in one small numbers were seen in stained films of the exudate. In three instances other bacteria which are normal inhabitants of the upper respiratory tract of monkeys were also present.

Two monkeys (Nos. 157 and 159) developed a clinically recognizable bronchopneumonia on the 3rd and 4th days respectively. The onset of pneumonia was insidious and was suspected only because of a rise in temperature and a moderate acceleration of the respiratory rate. Both animals were killed early in the course of the pneumonia and *Bacillus influenzæ* was recovered in pure culture from the lungs. A third monkey (No. 142) developed a small patch of bronchopneumonia which was not recognized during life. The pathology of the disease and its complications are described in the following paper.[18]

Experiment 1.—Monkey 150 (Text-fig. 1, *a*). *Cebus capucinus*, male; weight 1,350 gm. Aug. 23 and 24, 1919. Well and active. Aug. 25, 10.30 a.m. 1 cc. of 10 hour blood broth culture of *B. influenzæ* (Strain B. 11.11[2]) instilled into each nostril and 1 cc. into mouth with pipette. 2 p.m. Appears very sick and stuporous, lying curled up in corner of cage, paying little attention to surroundings; eyes dull; blinks lids frequently; presses hands on top of head; sneezes occasionally. 4.30 p.m. Condition the same; blood culture, no growth. 8 p.m. Sneezes very frequently. Aug. 26, 11 a.m. Still sick but less prostrated; refuses food; sneezes frequently; moderate mucoid nasal discharge; throat injected; stained film of nasal secretion shows many *B. influenzæ*, mostly phagocytosed; throat culture, *B. influenzæ*, non-hemolytic streptococci. 8 p.m. Condition the same. Aug. 27. Still sick, but appears better; sneezes occasionally; coughs occasionally; respirations normal. Aug. 28. Improving; sneezes and coughs occasionally. 10 a.m. Killed.

Autopsy. Anatomical Diagnosis.—Acute rhinitis; acute tracheobronchitis; hyperplasia of thymus.

Cultures.—Heart's blood, no growth; nasal mucous membranes, *Staphylococcus albus*; trachea, *B. influenzæ* and *Streptococcus viridans*.

Experiment 2.—Monkey 154 (Text-fig. 1, *b*). *Cebus capucinus*, male; weight 1,740 gm. Aug. 25, 1919. Well and active. Aug. 26, 11.15 a.m. Mucous membranes of nose and throat swabbed with cotton applicator previously dipped in salt solution washings from peritoneal cavity of Passage Monkey 13b (Strain B. 11.13[9]). 1.30 p.m. Quiet; head drooping; eyes closed; sneezes occasionally. 4 p.m. Refuses food. 8 p.m. Sneezes very frequently. Aug. 27, 10 a.m. Quiet; sneezes very frequently. Moderate mucopurulent nasal discharge; stained film shows many polymorphonuclear leucocytes, few *B. influenzæ*, and Gram-positive cocci. 8 p.m. Condition the same; shakes head and rubs nose frequently. Aug. 28. Appears better; sneezes frequently and coughs occasionally. Aug. 29. Appears weak and exhausted; coughs frequently. Killed.

Autopsy. Anatomical Diagnosis.—Acute rhinitis; acute tracheobronchitis; hyperplasia of thymus.

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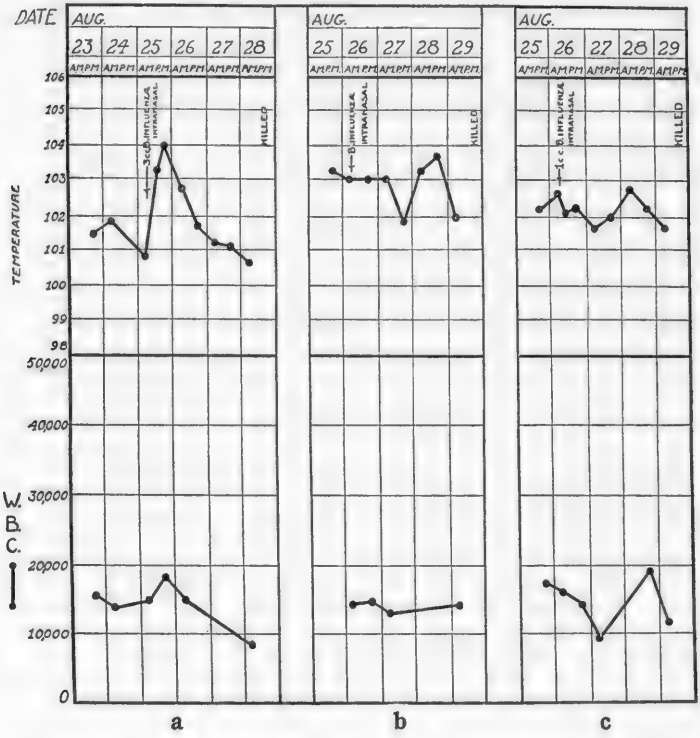
[18] Cecil, R. L., and Blake, F. G., *J. Exp. Med.*, 1920, xxxii, 719.

Cultures.—Heart's blood, no growth; trachea, *B. influenzæ*, *Streptococcus viridans*, and Gram-negative micrococcus.

Experiment 3.—Monkey 153 (Text-fig. 1, *c*). *Cebus capucinus*, female; weight 950 gm. Aug. 25, 1919. Well and active. Aug. 26, 11 a.m. 0.5 cc. of salt solution washings from peritoneal cavity of Passage Monkey 13b (Strain B. 11.13[0])



TEXT-FIG. 1, *a* to *c*. Acute respiratory disease produced by inoculation of the mucous membranes of the upper respiratory tract with *B. influenzæ*. (*a*) Monkey 150. (*b*) Monkey 154. (*c*) Monkey 153; complicated by sinusitis.

instilled into each nostril with pipette. 1.30 p.m. Appears stuporous; head drooping. 4 p.m. Very sick; lying prostrate on floor of cage; refuses food. 8 p.m. Sneezes occasionally. Aug. 27. Still sick but appears better; sneezes frequently. Moderate mucoid nasal discharge; stained film shows moderate number of polymorphonuclear leucocytes, lymphocytes, and epithelial cells but

no bacteria. Aug. 28. Quiet; head drooping; eyes closed much of time; sneezes frequently. Aug. 29. Still sick; no animation; sneezes and coughs occasionally. Killed.

Autopsy. Anatomical Diagnosis.—Acute rhinitis; acute purulent sinusitis, left antrum; acute tracheobronchitis; hyperplasia of thymus.

Stained Films.—Tracheal exudate, many leucocytes and epithelial cells, few *B. influenzæ*; left antrum, abundant leucocytes, few *B. influenzæ.*

Cultures.—Heart's blood, trachea, and left antrum, no growth.

In the following experiment the effect of exposure to cold and wet on the subsequent course of the disease was studied. Monkeys 148 and 139 were exposed to cold and wet for 4 hours after inoculation by sprinkling them with cold water from time to time and by directing a current of air on them with an electric fan. Monkeys 140 and 142, not subjected to exposure, served as controls.

Experiment 4.—Monkey 140 (Text-fig. 2, *a*). *Cebus capucinus*, female; weight 1,600 gm. Aug. 23 to 25, 1919. Well and active. Aug. 26, 10.10 a.m. Mucous membranes of nose and throat swabbed with cotton applicator previously dipped in peritoneal exudate of Passage Monkey 13b (Strain B. 11.13^0). 1.30 p.m. Very quiet. 4 p.m. Quiet; head drooping; sneezes occasionally. Aug. 27. Moderately sick; refuses food; sneezes occasionally. Scanty mucoid nasal discharge; stained film shows leucocytes and epithelial cells but no bacteria; culture, no *B. influenzæ*. Aug. 28. Appears sicker; lying down; very drowsy; coughs and sneezes frequently. Aug. 29. Appears better but is quiet and exhausted; sneezes occasionally; coughs frequently. Killed.

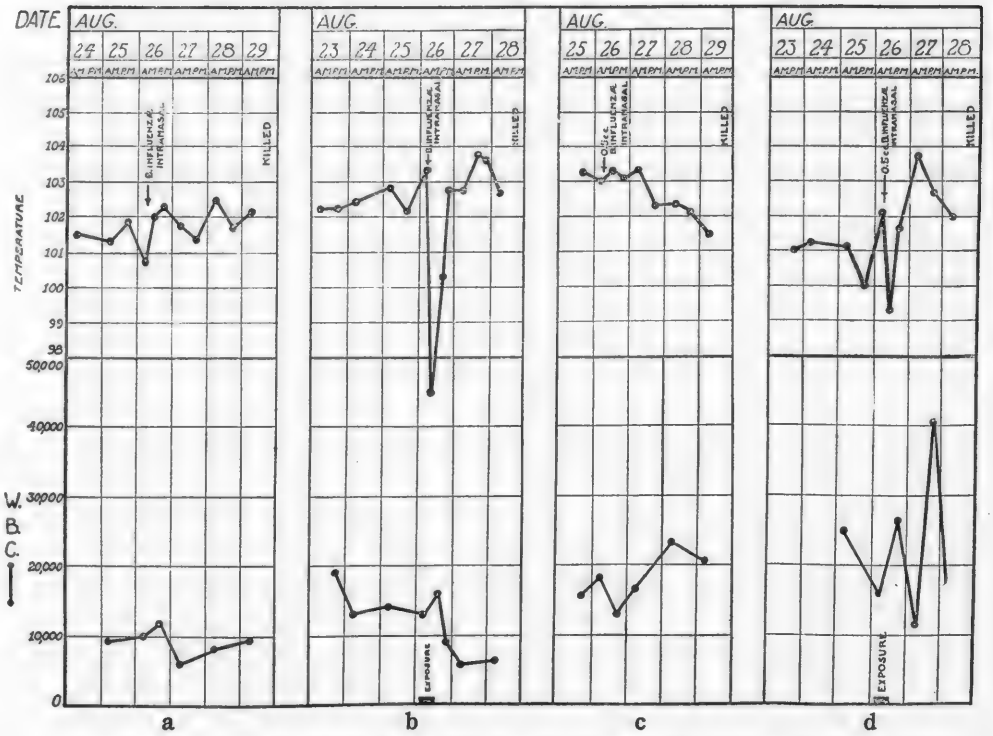
Autopsy. Anatomical Diagnosis.—Acute rhinitis; acute tracheobronchitis.

Cultures.—Heart's blood, no growth; nasal mucous membranes, *Staphylococcus albus* and large Gram-negative bacillus; trachea, *B. influenzæ*, *Staphylococcus albus*, and *Streptococcus viridans.*

Monkey 148 (Text-fig. 2, *b*). *Cebus capucinus*, female; weight 2,160 gm. Aug. 23 to 25, 1919. Well and active. Aug. 26, 10.20 a.m. Mucous membranes of nose and throat swabbed with cotton applicator previously dipped in peritoneal exudate of Passage Monkey 13b (Strain B. 11.13^0). 11 a.m. to 3 p.m. Exposure to cold and wet. 1.30 p.m. Very sick and stuporous, head drooping; eyes closed; shivering. 4 p.m. Prostrated; offers no resistance on handling; refuses food. Aug. 27. Very sick and prostrated; sneezes occasionally. Mucopurulent nasal secretion shows many leucocytes and epithelial cells and a few phagocytosed *B. influenzæ*. Aug. 28. Very sick; lying prostrate; sneezes frequently; no cough. Killed.

Autopsy. Anatomical Diagnosis.—Acute rhinitis; hyperplasia of thymus.

Cultures.—Heart's blood, trachea, and lungs, no growth; nasal mucous membranes, abundant *B. influenzæ* and few *Staphylococcus albus.*



TEXT-FIG. 2, *a* to *d*. Acute respiratory disease produced by inoculation of the mucous membranes of the upper respiratory tract with *B. influenzæ*. (*a*) Monkey 140. (*b*) Monkey 148. (*c*) Monkey 142; complicated by sinusitis and bronchopneumonia. (*d*) Monkey 139; complicated by sinusitis.

Monkey 142 (Text-fig. 2, *c*). *Cebus capucinus*, female; weight 1,300 gm. Aug. 23 to 25, 1919. Well and active. Aug. 26, 10.30 a.m. 0.25 cc. of peritoneal exudate from Passage Monkey 13b (Strain B. 11.13^0) instilled into each nostril with pipette. 1.30 p.m. Quiet; curled up in corner of cage; head drooping; eyes closed. 4 p.m. Quite sick and stuporous; refuses food; sneezes frequently. Aug. 27. Moderately sick; sneezes frequently. Mucopurulent nasal discharge shows many leucocytes and epithelial cells; no bacteria in stained film. Aug. 28. Still sick; sneezes very frequently. Aug. 29. Appears weak and without animation; sneezes occasionally. Killed.

Autopsy. Anatomical Diagnosis.—Acute rhinitis; acute sinusitis, left antrum; bronchopneumonia, right lower lobe.

Cultures.—Heart's blood and trachea, no growth; left antrum, few *Streptococcus viridans*.

Monkey 139 (Text-fig. 2, *d*). *Cebus capucinus*, female; weight 1,405 gm. Aug. 23 to 25, 1919. Well and active. Aug. 26, 10.40 a.m. 0.25 cc. of peritoneal exudate from Passage Monkey 13b (Strain B. 11.13^0) instilled into each nostril with pipette. 11 a.m. to 3 p.m. Exposure to cold and wet. 4 p.m. Very sick; head drooping; stuporous; eyes closed; shivering; refuses food. Aug. 27. Very sick; lying down; sneezes occasionally. Profuse mucopurulent nasal discharge showing abundant leucocytes, many *B. influenzæ*, and few staphylococci; culture, *B. influenzæ* and *Staphylococcus albus*. Aug. 28. Quite sick and stuporous; sneezes frequently and rubs nose; mucopurulent nasal discharge continues. 1.30 p.m. Killed.

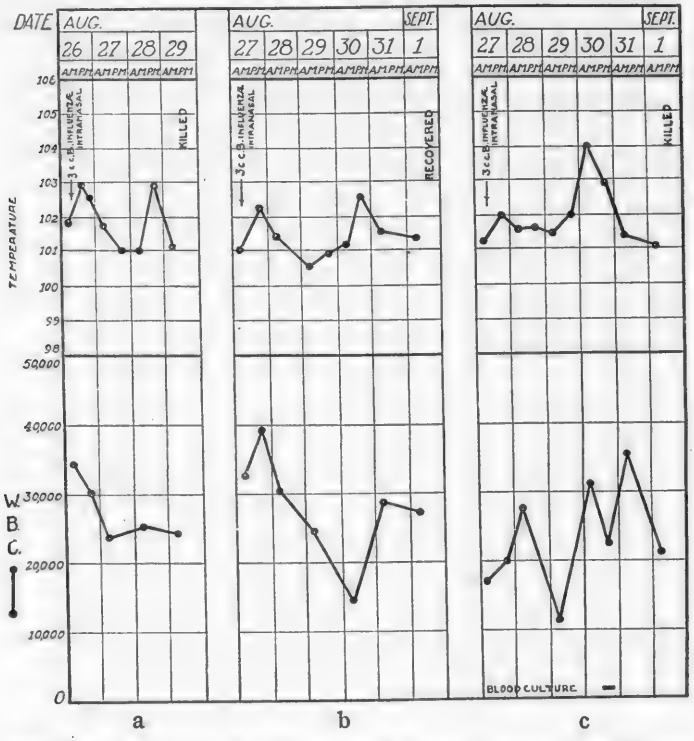
Autopsy. Anatomical Diagnosis.—Acute rhinitis; acute purulent sinusitis, both antra; acute tracheobronchitis; hyperplasia of thymus.

Cultures.—Heart's blood and trachea, no growth; both antra, abundant *B. influenzæ*.

Experiment 5.—Monkey 157 (Text-fig. 3, *a*). *Macacus syrichtus*, male; weight 5,200 gm. Aug. 25, 1919. Well, active, and ferocious. Aug. 26, 11.25 a.m. 1 cc. of salt solution washings of peritoneal cavity of Passage Monkey 13b (Strain B. 11.13^0) instilled into each nostril and 1 cc. into mouth. 2.30 p.m. Quite sick; lying down; stuporous; eyes closed. 4 p.m. Very sick; head drooping; refuses food. 10 p.m. Vomited. Aug. 27. Sick, no fight. Moderate mucopurulent nasal discharge; stained films show many polymorphonuclear leucocytes, many of them containing from two to twelve phagocytosed *B. influenzæ*; culture, no *B. influenzæ*. 2 p.m. Coughing violently; respirations not accelerated. Aug. 28. Still sick; sitting huddled up in corner of cage paying no attention to surroundings; coughs frequently; respirations moderately accelerated. Aug. 29. No animation; respirations accelerated; frequent cough. 11 a.m. Killed.

Autopsy. Anatomical Diagnosis.—Acute rhinitis; acute bilateral sinusitis of antra; acute tracheobronchitis; hemorrhagic edema of lungs; bronchiolitis; bronchiectasis; bronchopneumonia, left upper and right upper and lower lobes; hyperplasia of thymus.

Cultures.—Heart's blood and trachea, no growth; nasal mucous membrane, *B. influenzæ* and *Staphylococcus albus;* right antrum, *B. influenzæ* and Gram-negative micrococcus; right and left bronchi and right lower and upper lobes, pure culture of *B. influenzæ*; left upper lobe, *Streptococcus viridans.*



TEXT-FIG. 3, *a* to *c*. Acute respiratory disease produced by inoculation of the mucous membranes of the upper respiratory tract with *B. influenzæ*. (*a*) Monkey 157; complicated by sinusitis and bronchopneumonia. (*b*) Monkey 158. (*c*) Monkey 159; complicated by sinusitis and bronchopneumonia.

Experiment 6.—Monkey 158 (Text-fig. 3, *b*). *Macacus syrichtus*, male; weight 5,675 gm. Aug. 26, 1919. Well and ferocious. Aug. 27, 11 a.m. 3 cc. of 15 hour blood broth culture of *B. influenzæ* (Strain B. 11.13[3]) instilled into nose and mouth with pipette. 4 p.m. Quiet. Aug. 28. Appears moderately sick but without definite symptoms. Aug. 29 and 30. Condition the same; has developed a tight racking cough. Aug. 31. More active; coughs. Sept. 1. Appears well and active, but coughs occasionally. Observation discontinued.

Experiment 7.—Monkey 159 (Text-fig. 3, *c*). *Macacus syrichtus*, male; weight 5,430 gm. Aug. 26, 1919. Well, active, and ferocious. Aug. 27, 11.15 a.m. 3 cc. of 15 hour blood broth culture of *B. influenzæ* (Strain B. 11.13[3]) instilled into nose and mouth with pipette. 2 p.m. Appears sick; resting against side of cage. Aug. 28. Quiet. Mucoid nasal discharge; stained films show many leucocytes, some containing two to four *B. influenzæ*. Aug. 29. Has developed a dry racking cough; nasal discharge continues. 5 p.m. Appears quite sick; no animation; leaning against side of cage. Aug. 30 and 31. Condition the same; rubs eyes and nose and sneezes occasionally; coughs frequently; respirations accelerated. Sept. 1. Sick and exhausted; breathing accelerated; sneezes and coughs. 10.30 a.m. Killed.

Autopsy. Anatomical Diagnosis.—Acute rhinitis; acute bilateral sinusitis of antra; acute tracheobronchitis; hemorrhagic edema of lungs; bronchiolitis; bronchopneumonia, right upper and left upper, middle, and lower lobes; hyperplasia of thymus.

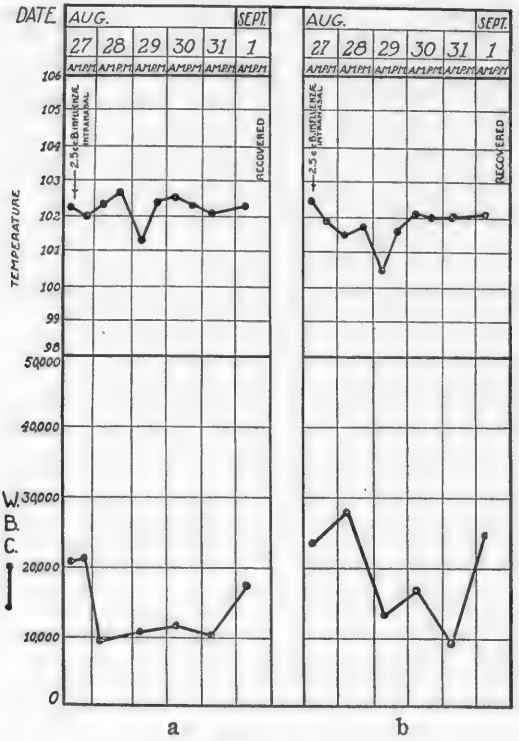
Cultures.—Heart's blood and left middle lobe, no growth; right antrum, pure culture of *B. influenzæ*; left antrum, *B. influenzæ* and *Streptococcus viridans*; trachea, *B. influenzæ*, *Streptococcus viridans*, and Gram-negative micrococcus; right and left bronchi, *B. influenzæ* and Gram-negative micrococcus; left lower and upper lobes, pure culture of *B. influenzæ*.

Experiment 8.—Monkey 160 (Text-fig. 4, *a*). *Cebus capucinus*, male; weight 1,500 gm. Aug. 26, 1919. Well and active. Aug. 27, 11.25 a.m. 2.5 cc. of 15 hour blood broth culture of *B. influenzæ* (Strain B. 11.13[3]) instilled into nose and mouth with pipette. 3 p.m. Sick; lying flat; rubs eyes; sneezes frequently. Aug. 28. Sick but less prostrated; sneezes and rubs eyes and nose frequently. Mucoid nasal discharge; stained film shows leucocytes and epithelial cells but no bacteria. Aug. 29. Condition the same. 3 p.m. Has begun to cough. Aug. 31. Appears better; sneezes and coughs occasionally. Sept. 1. Appears well. Observation discontinued.

Experiment 9.—Monkey 161 (Text-fig. 4, *b*). *Cebus capucinus*, male; weight 1,340 gm. Aug. 26, 1919. Well and active. Aug. 27, 11.35 a.m. 2.5 cc. of 15 hour blood broth culture of *B. influenzæ* (Strain B. 11.13[3]) instilled into nose and mouth with pipette. 1 p.m. Begins to appear sick. 2 p.m. Very sick; lying prostrate. 4 p.m. Lying down; stuporous; eyes closed; sneezes frequently. Aug. 28. Sick though less prostrated; mucopurulent nasal discharge; sneezes frequently. Aug. 29 and 30. Condition the same; coughs occasionally. Aug. 31. Appears better; coughs and sneezes occasionally. Sept. 1. Improving; observation discontinued.

In order to determine whether the culture medium in which *Bacillus influenzæ* had grown would exert any toxic effect when inoculated on the mucous membranes of the upper respiratory tract the following experiment was carried out.

Experiment 10.—Monkey 156. *Cebus capucinus*, female; weight 1,120 gm. Aug. 26, 1919. Well and active. 3 cc. of supernatant broth of a 16 hour blood broth culture of *B. influenzæ* (Strain B. 11.13[2]) freed from bacteria by centrifugalization instilled into nose and mouth with pipette. Monkey remained well and active, exhibiting no symptoms throughout a period of 6 days when observation was ended.



TEXT-FIG. 4, *a* and *b*. Acute respiratory disease produced by inoculation of the mucous membranes of the upper respiratory tract with *B. influenzæ*. (*a*) Monkey 160. (*b*) Monkey 161.

Effect of Intratracheal Injection of Bacillus influenzæ.

Ten monkeys were injected intratracheally with *Bacillus influenzæ* of enhanced virulence (Table III). These experiments may properly be divided into two groups of five animals each. In the first group

(Monkeys 124, 20, 137, 138, and 149) massive doses of *Bacillus influenzæ* were employed in an attempt further to increase the virulence of the organism for the respiratory tract of monkeys if possible. This did not prove feasible, however, and was abandoned. Three of these animals developed tracheobronchitis and bronchopneumonia, one bronchitis and bronchopneumonia, and one tracheobronchitis without pneumonia. In one instance (Monkey 138) *Bacillus influenzæ* pericarditis and septicemia ensued. Although all these animals were unquestionably infected with *Bacillus influenzæ* the value of the results is lessened because of the large doses employed. This is shown by the fact that a control monkey (No. 155) injected intratracheally with an equivalent amount of *Bacillus influenzæ* previously killed by heating at 56°C. for 1 hour, although showing none of the other evidences of actual infection exhibited by the monkeys inoculated with living cultures, did show moderate bronchopneumonic lesions at autopsy. In the second group (Monkeys 108, 136, 135, 141, and 152) a comparatively small dose (1 cc.) was employed with the successful production of bronchopneumonia in three animals. One developed tracheobronchitis without pneumonia and one resisted infection. In this group the development of pneumonia was clearly dependent upon actual infection with *Bacillus influenzæ*.

The course and general symptoms of the disease produced in these animals were similar to those occurring in the monkeys inoculated in the upper respiratory tract; that is, a self-limited respiratory disease of 3 to 6 days duration, sudden onset after a brief incubation period, prostration, a variable febrile reaction, and leucopenia, or no significant change in the leucocyte count. Leucocytosis with pericarditis occurred in Monkey 138. The disease was accompanied by a frequent and severe racking cough and accelerated respiratory rate. In no case did the disease prove fatal. It seems probable, however, that death would have occurred in the monkey that developed pericarditis and septicemia had it not been killed.

The nine monkeys showing clinical evidence of infection were killed at varying intervals after inoculation for pathological and bacteriological examination. In three of the seven monkeys which developed pneumonia *Bacillus influenzæ* was recovered in pure culture from the lungs or bronchi, in four the cultures remained sterile, indicating that

TABLE III.

Effect of Intratracheal Injection of B. influenzæ.

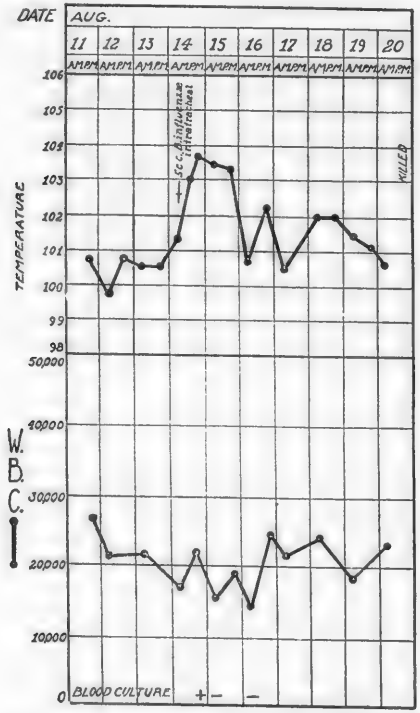
Monkey No.	Species.	Weight.	Date of injection.	Material injected.	Strain of *B. influenzæ* injected.	Amount injected.	Result.	Autopsy cultures.
		gm.	*1919*			*cc.*		
124	*M. syrichtus.*	1,500	Aug. 11	Broth suspension of 16 hr. plate culture.	B. 11.7[2]	5.0	Tracheobronchitis; bronchopneumonia. Killed on 3rd day.	H. B., n. g. Tr., B. I. Lungs, B. I.
20	" "	3,500	" 14	Broth suspension of 12 hr. plate culture.	" 11.8[2]	5.0	Tracheobronchitis. Recovered by 7th day. Killed.	H. B., n. g. Tr., B. I.
137	" "	2,830	" 19	" "	" 11.10[2]	4.0	Tracheobronchitis; bronchopneumonia. Killed on 3rd day.	H. B., n. g. Tr., " Lungs, "
138	" "	2,915	" 19	" "	" 11.10[2]	4.0	Tracheobronchitis; bronchopneumonia; acute pericarditis; septicemia. Killed on 3rd day.	H. B., B. I. Tr., " Lungs, " Pericard., "
149	*C. capucinus.*	1,500	" 23	" "	" 11.11[2]*	4.0	Bronchiolitis; bronchopneumonia. Recovered by 6th day. Killed.	H. B., n. g. Tr., " Lungs, "
108	*M. syrichtus.*	2,230	" 14	" "	" 11.8[2]	1.0	Tracheobronchitis. Recovered by 6th day. Killed.	H. B., n. g. Tr., "
136	*C. capucinus.*	1,100	" 14	Peritoneal exudate of Monkey P 8.	" 11.8[0]	1.0	Tracheobronchitis; hemorrhagic edema of lungs; bronchopneumonia. Killed on 6th day.	H. B., " Br., B. I. Lungs, n. g.
135	*M. syrichtus.*	2,150	" 14	Peritoneal exudate of Monkey P 9.	" 11.9[0]	1.0	No disease.	

141	*C. capucinus.*	1,400	Aug. 22	Broth suspension of 15 hr. plate culture.	B. 11.11[1]*	1.0	Tracheobronchitis; bronchopneumonia; convalescent by 8th day. Killed.	H. B., n. g. Tr., " Turb., Staph. A.
152	" "	1,035	" 26	Peritoneal exudate of Monkey P 13b.	" 11.13[0]	1.0	Tracheobronchitis; bronchopneumonia. Killed on 2nd day.	H. B., n. g. Tr., " Lungs, "

* This culture was a strain of *B. influenzæ* recovered from Monkey 138 at autopsy.

H. B. indicates heart's blood; Tr., trachea; Br., bronchi; turb., turbinates; B. I., *B. influenzæ*; n. g., no growth; Staph. A., *Staphylococcus albus*.

the infection had terminated. In the two animals in which only a tracheobronchitis developed, cultures from the trachea at autopsy yielded *Bacillus influenzæ* in pure culture in one, no growth in the other. Other bacteria, normal inhabitants of the upper respiratory tract of monkeys, were not found in this series. Their total absence



TEXT-FIG. 5. Monkey 20. Tracheobronchitis following intratracheal injection of *B. influenzæ*.

was in striking contrast with their frequent presence in the trachea in the series of monkeys inoculated on the mucous membranes of the upper respiratory tract in which it appeared that the spread of the influenza bacillus infection from the upper to the lower respiratory tract permitted the ready entrance of secondary organisms into the lower respiratory passages. Cultures of the heart's blood remained

sterile except in one instance in which *Bacillus influenzæ* was present in pure culture. The pathology of the disease is presented in the following paper.[18] Illustrative protocols follow.

Experiment 11.—Monkey 20 (Text-fig. 5). *Macacus syrichtus*, male; weight 3,500 gm. Aug. 11 to 13, 1919. Well and active. Aug. 14, 10.30 a.m. Intratracheal injection of 5 cc. of broth suspension (five plates) of 12 hour culture of *B. influenzæ* (Strain B. 11.8[2]). 4.15 p.m. Appears quite sick; resting against side of cage; breathing rapidly. 9.30 p.m. Very sick; lying down. Aug. 15. Sick; lying down or leaning against side of cage most of time; respirations moderately accelerated; frequent hard, racking cough. Aug. 16. Condition the same; cough continues. Aug. 17 and 18. Improving, but still sick and without appetite; appears weak and exhausted; coughs frequently. Aug. 19. Appears better and more active; breathing easily; no cough. Aug. 20. Appears well. 9.40 a.m. Killed.

Autopsy. Anatomical Diagnosis.—Acute tracheobronchitis; hyperplasia of thymus and thoracic lymph nodes.

Cultures.—Heart's blood, no growth; trachea, pure culture of *B. influenzæ*.

Experiment 12.—Monkey 136 (Text-fig. 6, *a*). *Cebus capucinus* male; weight 1,100 gm. Aug. 14, 1919. Well and active. 2.30 p.m. Intratracheal injection of 1 cc. of peritoneal exudate of Passage Monkey 8, 10 minutes after removal of exudate at autopsy. 5 p.m. Begins to appear sick. 7 p.m. Very sick; lying prostrate; stuporous. 9 p.m. Prostrate; offers no resistance on handling; respirations accelerated. Aug. 15. Sick but no longer prostrated; no appetite; coughs. Aug. 16. Sick; lying down most of time; coughs occasionally. Aug. 17. Appears better but is weak and exhausted; no appetite; respirations moderately accelerated. Aug. 18. Condition the same. Aug. 19. More active. 11 a.m. Killed.

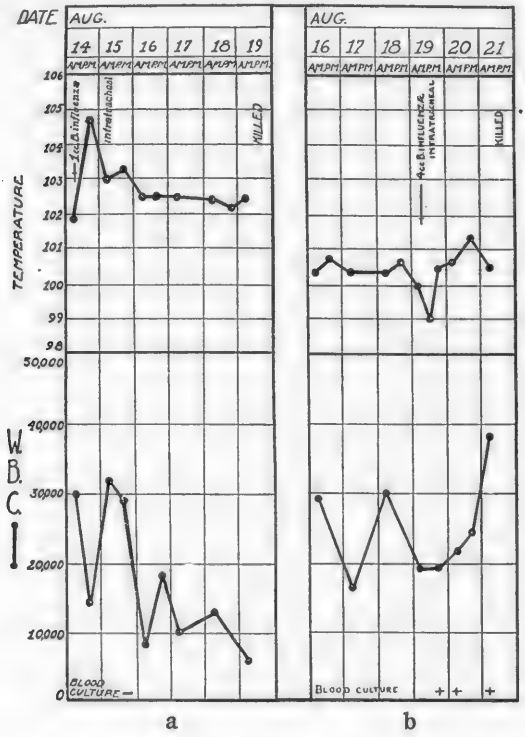
Autopsy. Anatomical Diagnosis.—Acute tracheobronchitis and bronchiolitis; hemorrhagic edema of lungs, all lobes; bronchopneumonia, all lobes; hyperplasia of thymus, spleen, and thoracic and abdominal lymph nodes.

Cultures.—Heart's blood and lungs, no growth; right bronchus, pure culture of *B. influenzæ*.

Experiment 13.—Monkey 138 (Text-fig. 6, *b*). *Macacus syrichtus*, female; weight 2,915 gm. Aug. 16 to 18, 1919. Well and active. Aug. 19, 10.45 a.m. Intratracheal injection of 4 cc. of broth suspension (four plates) of 12 hour culture of *B. influenzæ* (Strain B. 11.10[2]). 4.30 p.m. Sick; resting against side of cage; respirations very rapid. 7.30 p.m. Lying prostrate; groaning; coughs occasionally. Aug. 20. Prostrated; breathing rapidly; coughs. Aug. 21. Very sick. 10 a.m. Killed.

Autopsy. Anatomical Diagnosis.—Acute tracheobronchitis; lobular bronchopneumonia, right lower, left upper, and left lower lobes; acute pericarditis; chronic fibrous pleuritis, right side; hyperplasia of thymus and spleen.

Cultures.—Heart's blood, trachea, left lower lobe, and pericardium, pure culture of *B. influenzæ*.



TEXT-FIG. 6, *a* and *b*. Effect of intratracheal injection of *B. influenzæ*. (*a*) Monkey 136; tracheobronchitis, hemorrhagic edema of lungs, and bronchopneumonia. (*b*) Monkey 138; tracheobronchitis, bronchopneumonia, pericarditis, and septicemia.

DISCUSSION AND SUMMARY.

Twelve normal monkeys inoculated on the mucous membranes of the nose or nose and mouth with a strain of *Bacillus influenzæ* originally isolated in pure culture from the pleural exudate of a case of empyema following influenzal pneumonia in man and subsequently raised in virulence by animal passage, developed an acute self-limited

respiratory disease of from 3 to 5 days duration, characterized by sudden onset with profound prostration, the development of rhinitis and tracheobronchitis, with sneezing, cough, and the outpouring of a scanty mucoid, or mucopurulent exudate, a variable febrile reaction, and either a leucopenia or no significant change in the leucocyte count. This disease was complicated in five instances by purulent sinusitis of one or both antra, in three by bronchopneumonia. *Bacillus influenzæ* was recovered at autopsy from the lesions of the disease either in pure culture or in association with organisms that are normal inhabitants of the upper respiratory tract of monkeys.

Of ten normal monkeys injected intratracheally with the same strain of *Bacillus influenzæ*, seven developed bronchopneumonia, two developed tracheobronchitis without pneumonia, and one resisted infection. The general symptoms and duration of the disease were similar to those of the preceding group. There were a severe cough and accelerated respirations. *Bacillus influenzæ* was recovered in pure culture from the lungs, bronchi, or trachea in the animals killed during the active stage of the disease. It disappeared promptly from the respiratory tract with recovery.

The significance of the first series of experiments in which monkeys were inoculated in the upper respiratory tract is twofold. First, they establish the fact that *Bacillus influenzæ* can initiate in monkeys an acute infection of the normal mucous membranes of the upper respiratory tract; that is, it can act as a primary incitant of respiratory infection without the assistance of a preceding or concomitant contributing cause. In this respect it differs radically from the pneumococcus and *Streptococcus hæmolyticus*, since experiments previously reported[2, 4] have shown that neither of these organisms possesses the property of initiating an infection of the normal mucous membranes of the upper respiratory tract of monkeys, even though the strains used were incalculably more virulent for monkeys than the strain of *Bacillus influenzæ* used in the foregoing experiments. Secondly, the experiments show that *Bacillus influenzæ* infection of the mucous membranes of the upper respiratory tract may spread by continuity to the paranasal sinuses, setting up an acute sinusitis, that it spreads readily to the lower respiratory tract, producing a tracheobronchitis and permitting the ready invasion of secondary bacteria, and that it may

penetrate as far as the terminal bronchioles, alveolar ducts, atria, and alveoli, there setting up a bronchiolitis and true bronchopneumonia. In these respects it likewise differs radically from the pneumococcus and *Streptococcus hæmolyticus* which do not possess these pathogenic properties as previous experiments have shown.[2, 4]

The bearing of these facts on the possible etiologic relation of *Bacillus influenzæ* to influenza is important, since they show that *Bacillus influenzæ* possesses certain definite primary pathogenic properties which distinguish it and therefore separate it from the group of recognized secondary organisms in influenzal complications, of which the pneumococcus and the streptococcus are the most frequent. The possible etiologic relation of *Bacillus influenzæ* to influenza is further supported by the character of the respiratory disease that occurred in the monkeys. The sudden onset with profound prostration, the absence of leucocytosis or often a leucopenia, the congestion of the mucous membranes of the respiratory tract, the development on the 2nd or 3rd day of an irritative cough due to an inflammatory tracheitis or tracheobronchitis, the brief self-limited course of the infection, and the irregular febrile reactions are all characteristic of influenza. Many of these symptoms were in striking contrast with the symptoms and course of pneumococcus or streptococcus infections in monkeys in which there were no prostration at onset, invariable leucocytosis, and infrequent cough developing only late in the disease.

While all the above features of the disease produced in monkeys are characteristic of influenza in man, none are pathognomonic and, in fact, it is doubtful whether uncomplicated influenza possesses any pathognomonic features by which it may be diagnosed certainly in the absence of an epidemic. Even during epidemic times many respiratory infections arise which, though presumably influenza, it is impossible to diagnose as such with certainty. Nor does pathology help in this respect, since there would appear to be no established distinctive lesions of uncomplicated influenza in man, nor for that matter of the complications of influenza, apart from the complications which have been ascribed by Pfeiffer,[5] MacCallum,[6] Wolbach,[7] and others to infection with *Bacillus influenzæ* because of the association of *Bacillus influenzæ* in pure culture with these complications. For these reasons, although the disease produced in monkeys appears

to be essentially identical with influenza in man with respect to its clinical course and complications, it is impossible to determine certainly whether it is actually so. The experiments are advanced, therefore, as evidence in favor of the etiologic relation of *Bacillus influenzæ* to influenza, though they do not permit of a definite conclusion in this respect.

Their bearing upon the relation of *Bacillus influenzæ* to certain of the complications of influenza would appear to be reasonably conclusive. The recovery of *Bacillus influenzæ* in pure culture at autopsy from the antra, from the trachea and bronchi, and from the lungs in some of the animals developing sinusitis, bronchiolitis, and a characteristic type of bronchopneumonia confirms by animal experiment the etiologic relation of *Bacillus influenzæ* to these complications of influenza, which hitherto has rested solely upon the frequent association of the influenza bacillus with these lesions in man. The production of tracheobronchitis and the same type of bronchopneumonia by the intratracheal injection of *Bacillus influenzæ* in the second series of experiments serves as additional confirmation of this, but has no direct bearing on the etiologic relation of *Bacillus influenzæ* to uncomplicated influenza.

CONCLUSIONS.

1. *Bacillus influenzæ* can initiate in normal monkeys an acute infection of the upper respiratory tract which may be complicated by acute sinusitis, tracheobronchitis, hemorrhagic edema of the lungs, bronchiolitis, and bronchopneumonia.

2. This disease appears to be essentially identical with influenza with respect to its clinical course, symptoms, and complications.

3. The etiologic relation of *Bacillus influenzæ* to acute sinusitis, tracheobronchitis, bronchiolitis, and bronchopneumonia is established.

4. Although it seems reasonable to infer from the results of the experiments that *Bacillus influenzæ* is the specific cause of influenza, a definite conclusion is not permissible, since it is impossible to determine whether the respiratory disease produced in monkeys with *Bacillus influenzæ* is identical with influenza or merely similar to it. The experiments are advanced, therefore, as evidence in favor of the etiologic relation of *Bacillus influenzæ* to influenza.

STUDIES ON EXPERIMENTAL PNEUMONIA.

X. Pathology of Experimental Influenza and of Bacillus influenzæ Pneumonia in Monkeys.

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Plates 90 to 97.

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The observation has frequently been made that influenza, as it occurs in man, possesses no characteristic pathology. The disease usually manifests itself primarily as a profound intoxication accompanied by a catarrhal inflammation of the upper respiratory tract, which, when uncomplicated, is rarely fatal. So far as known, the inflammatory changes display no properties which differentiate them from those occurring with other types of infection in the same locality. Further confusion is added to the pathology of influenza by reason of the mixed bacteriological findings. While *Bacillus influenzæ* can usually be isolated from the secretions, it is generally found in conjunction with other bacteria, such as streptococcus or pneumococcus. This lack of distinctive features in the pathology of influenza has greatly enhanced the difficulty of identifying the disease in man and of studying it experimentally in animals. None of the earlier investigators made any claims to having transmitted influenza to animals.

Pfeiffer[1] carried out many experiments on various laboratory animals, including monkeys, injecting them intratracheally, intravenously, and directly into the lung tissue, but always without positive results. If large doses of culture were administered the animals usually showed symptoms of toxemia and sometimes died; but he was never able to demonstrate an actual multiplication of the influenza bacilli—a true infection.

In respect to the pathology of *B. influenzæ* pneumonia, accurate knowledge has been almost as difficult to obtain as in influenza. Wollstein and Meltzer[2]

[1] Pfeiffer, R., *Z. Hyg. u. Infectionskrankh.*, 1893, xiii, 357.

[2] Wollstein, M., and Meltzer, S. J., *J. Exp. Med.*, 1912, xvi, 126.

succeeded in producing patches of consolidation in the lungs of dogs by insufflating massive doses of influenza bacilli directly into the bronchi, but with this exception, *B. influenzæ* pneumonia has never been reproduced in animals; and pure *B. influenzæ* pneumonia in man is so rarely encountered that it has been hard to say which of the lesions are due to the influenza bacillus, and which to the other bacteria present. Although *B. influenzæ* pneumonia is usually characterized by the presence of other bacteria, notably the streptococcus and pneumococcus, in addition to the influenza bacillus, pure *B. influenzæ* infections occur, and several investigators have described the pathological picture in such cases (Pfeiffer,[1] MacCallum,[3] and Wolbach[4]).

The descriptions by these writers of the pathology of *B. influenzæ* pneumonia in man are quite similar and indicate clearly that pure *B. influenzæ* infections of the lung produce a characteristic group of lesions. Briefly, these authors describe the condition as follows: Dark red, partially air-containing lungs; slight if any pleural exudate; on section, edema and hemorrhage; purulent bronchitis and bronchiolitis; peribronchial infiltration and thickening; bronchopneumonia; bronchiectasis and emphysema. Microscopically serous and hemorrhagic exudate; dilatation of bronchioles and alveoli; partial destruction of bronchial mucous membrane; pus in bronchi and bronchioles; round cell infiltration and thickening of bronchial walls; infiltration of leucocytes in neighboring alveolar walls; leucocytes and desquamated epithelium in adjacent alveoli; deposit of hyaline material on alveolar walls; organization.

In the preceding article[5] it has been shown that by inoculating monkeys intranasally with a virulent culture of *Bacillus influenzæ*, an infection of the upper respiratory tract may be induced which is clinically comparable to influenza in man, and furthermore, that intratracheal injections of virulent influenza bacilli excite in monkeys a bronchopneumonia analogous to *Bacillus influenzæ* pneumonia in man. In the present paper the pathological findings of experimental *Bacillus influenzæ* respiratory infections will be discussed in detail.

As influenza in man is practically never a fatal disease unless complicated by pneumonia, there has been little or no opportunity for studying the general pathology of the disease. There is no reason to believe, however, that simple influenza is regularly accompanied by

[3] MacCallum, W. G., *J. Am. Med. Assn.*, 1919, lxxii, 720; The pathology of the pneumonia in the United States Army camps during the winter of 1917–18, Monograph of The Rockefeller Institute for Medical Research, No. 10, New York, 1919.

[4] Wolbach, S. B., *Bull. Johns Hopkins Hosp.*, 1919, xxx, 104.

[5] Blake, F. G., and Cecil, R. L., *J. Exp. Med.*, 1920, xxxii, 691.

extensive pathological changes in the various internal organs. It appears to be a local infection of the upper respiratory tract, the general symptoms of such infection probably being referable to absorption of toxic substances. Briefly, it may be said that uncomplicated influenza in man is characterized by swelling and hyperemia of the nasal and pharyngeal mucous membrane with a variable amount of mucoid discharge which later may become profuse and mucopurulent, and usually by acute inflammatory changes in the trachea and larger bronchi. Injection of the conjunctivæ and flushing of the face are also frequently seen. In uncomplicated cases there is little evidence pointing to significant pathological lesions of the internal organs.

Pathology of Experimental Influenza in Monkeys.

In the preceding paper the method of producing experimental influenza in monkeys has been described in detail, and will, therefore, not be repeated here.

In Table I the monkeys that were infected by the inoculation of virulent influenza bacilli into the nose and throat are presented, with the dose injected and the pathological findings in each. Three of these monkeys (Nos. 158, 160, and 161) were not killed. They presented, however, all the clinical signs of acute rhinitis and acute tracheitis. The remaining nine monkeys were killed during, or immediately following their infection, and were subjected at once to complete autopsy.

Rhinitis.—By referring to Table I it will be seen that at autopsy each of the nine monkeys showed acute rhinitis, and that in five out of the nine *Bacillus influenzæ* was recovered by culture or film from some part of the upper respiratory tract (Nos. 148, 139, 153, 157, and 159). The nasal mucous membrane in these nine monkeys presented very much the same appearance. The mucosa was swollen and intensely reddened, especially over the turbinates. The blood vessels were congested, and the mucous membrane was covered with a mucoid or mucopurulent exudate. In short, the process was analogous to acute rhinitis in man.

Microscopic sections from the nasal mucous membrane show an exudate of mucus, pus cells, and desquamated epithelial cells on the

TABLE I.

Monkeys That Received B. influenzæ in the Nose and Throat.

Monkey No.	Date of inoculation.	Amount inoculated.		Date killed.	Pathological findings.	Autopsy cultures.
		Nose.	Throat.			
	1919	*cc.*	*cc.*	*1919*		
150	Aug. 25	2.0	1.0	Aug. 28	Rhinitis; tracheobronchitis; hyperplasia of thymus.	Nasal m. m., Staph. A. Tr., B. I.; Str. V.
140	" 26	Swab.	Swab.	" 29	Rhinitis; tracheobronchitis.	Turb., Staph. A.; large Gr.-neg. bac. Tr., B. I; Staph. A.; Str. V.
148	" 26	"	"	" 28	" hyperplasia of thymus.	Turb., B. I.; Staph. A. Tr., n. g. Lungs, n. g.
142	" 26	0.5		" 29	" sinusitis of left antrum; bronchopneumonia, R. L.	Left antrum, Str. V. Tr., n. g.
139	" 26	0.5		" 28	Rhinitis; bilateral sinusitis; tracheobronchitis; hyperplasia of thymus.	Right antrum, B. I. pure. Left " " " Tr., n. g.
153	" 26	1.0		" 29	Rhinitis; tracheobronchitis; sinusitis of left antrum; hyperplasia of thymus and cervical lymph glands.	Left antrum, n. g.; few B. I. in films. Tr., n. g.; few B. I. in films.
154	" 26	Swab.	Swab.	" 29	Rhinitis; tracheobronchitis; hyperplasia of thymus and cervical lymph glands.	" B. I.; Str. V.; Gr.-neg. micr.

157	Aug. 26	2.0	1.0	Aug. 29	Rhinitis; tracheobronchitis; bilateral sinusitis of antra; hemorrhagic edema of lungs and bronchopneumonia, R. U., L. U., R. L.; hyperplasia of thymus and thoracic lymph glands; bronchiectasis; emphysema.	Left turb., B. I.; Staph. A. Right antrum, B. I.; Gr.-neg. coccus. Tr., n. g. Both br., pure B. I. R. U., pure B. I. R. L., " " L. U., no B. I.; 6 col. Str. V.
158	" 27	2.0	1.0	Not killed.	(Rhinitis; tracheitis.)	
159	" 27	2.0	1.0	Sept. 1	" tracheobronchitis; bilateral sinusitis of antra; hemorrhagic edema of lungs; bronchopneumonia, L. U., L. M., R. U.; organizing pneumonia, L. L.; hyperplasia of thymus and thoracic lymph glands.	Tr., B. I.; few Gr.-neg. micr. and Str. V. Br., B. I.; Gr.-neg. micr. Left antrum, B. I.; Str. V. Right " pure B. I. L. U., pure B. I. L. M., n. g. L. L., pure B. I.
160	" 27	1.5	1.0	Not killed.	(Rhinitis; tracheitis.)	
161	" 27	1.5	1.0	Not killed.	" "	

R. L., L. L., etc., indicate lobes of the lung.

Br. indicates bronchus; m.m., mucous membrane; turb., turbinates; n. g., no growth; Staph. A., *Staphylococcus albus*; B. I., *B. influenzæ*; Str. V., *Streptococcus viridans*; Gr.-neg., Gram-negative. The other abbreviations are self-explanatory.

surface of the epithelium, which is eroded in some places (Fig. 1). The submucosa is engorged, and is infiltrated with polymorphonuclear leucocytes, many of which are also seen between the columnar epithelial cells. Goblet cells are numerous.

Tracheobronchitis.—In seven of the nine animals (Nos. 150, 140, 139, 153, 154, 157, and 159) the inflammation had extended from the nasopharynx down into the trachea and bronchi. Tracheobronchitis can hardly be called a complication in this instance, since it occurs in monkeys, as in man, in a large percentage of cases. In this group the trachea and bronchi were sometimes reddened, but more often the mucous membrane showed only an injection of the blood vessels and a deposit of mucus over the surface. Cultures from the trachea or large bronchi yielded *Bacillus influenzæ* in five out of seven cases (Nos. 140, 150, 154, 157, and 159). Microscopic sections from the trachea show acute inflammatory changes similar to those found in the nasal mucous membrane (Figs. 2 and 3).

Sinusitis.—Five of the nine cases that were autopsied showed, in addition to rhinitis, an infection of one or both of the antra of Highmore (Nos. 142, 139, 153, 157, and 159). *Bacillus influenzæ* infection of the antrum in monkeys is similar to that in man. The mucous membrane is reddened and swollen and the cavity is partially filled with mucopurulent exudate which in one instance was tinged with blood. Cultures from the antra showed influenza bacilli in three cases (Nos. 139, 157, and 159). Of the remaining two, one revealed influenza bacilli in films (Monkey 153) and the other (Monkey 142) yielded *Streptococcus viridans*, probably a secondary invader that had supplanted the influenza bacillus.

Hyperplasia of the Lymph Glands.—The cervical lymph glands were enlarged in two monkeys (Nos. 153 and 154). Microscopic sections show hyperplasia of the follicles, with an increased number of leucocytes in the sinuses.

Hyperplasia of the Thymus.—One of the most interesting changes observed in these monkeys was a well marked hyperplasia of the thymus gland. This condition was entirely unexpected, and so far as known has not been described in *Bacillus influenzæ* infections in man. In seven of the nine monkeys that were autopsied (Monkeys 150, 148, 139, 153, 154, 157, and 159) the thymus gland was definitely

enlarged, in some instances extending down for a considerable distance over the pericardium. The gland was rather firm and had a peculiar pinkish yellow, flesh-like appearance. The lobules were readily recognized. Microscopic sections show marked hyperplasia of the follicles, due to proliferation of the large cells composing the chyme centers. The lymphatic channels leading to the gland are greatly dilated and filled with coagulated plasma. Even the small channels in the center of the follicles are often dilated, and, in addition, contain a considerable number of polymorphonuclear leucocytes and eosinophils. In some instances the interstitial tissue between the lobules is densely infiltrated with leucocytes.

The following case of experimental influenza illustrates the lesions above described and is reported in full.

Protocol 1.—Monkey 139. *Cebus capucinus*, female; weight 1,405 gm. Aug. 26, 1919. Injected with 0.5 cc. of *B. influenzæ* peritoneal exudate intranasally. Aug. 28. Killed by blow on cervical vertebræ.

Autopsy.—Subject is a small brown Capuchin. Thymus shows moderate enlargement. Pericardium and pleura normal; heart is not enlarged; myocardium and valves normal. Trachea and bronchi contain a moderate quantity of mucus. Bronchial lymph glands are not enlarged. Nasal cavities contain a considerable amount of mucopurulent material. Turbinates on both sides are swollen and reddened and their vessels stand out prominently. The right antrum contains 10 or 12 drops of cloudy fluid; the mucous membrane is swollen and edematous. The left antrum also contains cloudy fluid and is similar in appearance to the right. The lungs are pale and voluminous and free from consolidation. Abdominal organs present nothing of importance.

Cultures.—Right antrum, pure culture of *B. influenzæ*; left antrum, pure culture of *B. influenzæ*; trachea, no growth.

Anatomical Diagnosis.—Acute rhinitis; acute bilateral sinusitis; acute tracheobronchitis; hyperplasia of thymus.

Microscopic Examination.—Trachea.—Blood and mucus on surface of epithelium, which shows many goblet cells. Vessels of submucosa engorged; infiltration of leucocytes beneath epithelium. *Turbinate.*—Mucous membrane covered with a thick layer of exudate composed of red blood cells, leucocytes, and desquamated epithelial cells (Fig. 1). The epithelium is eroded in many places. Numerous leucocytes are working their way out between the epithelial cells. There is infiltration of leucocytes in the submucosa. Blood vessels of submucosa are distended with red blood cells. *Lungs.*—Normal. *Thymus.*—Shows hyperplasia of follicles and distention of lymph channels with coagulated plasma.

Three of the monkeys in this group developed bronchopneumonia (Nos. 142, 157, and 159), which is a complication of such importance that it will be discussed in considerable detail.

Pathology of Bacillus influenzæ Pneumonia in Monkeys.

Bacillus influenzæ Pneumonia Developing Spontaneously in Monkeys with Experimental Influenza.

In three of the twelve monkeys inoculated in the nose and throat with *Bacillus influenzæ*, the infection in the upper respiratory tract extended down into the bronchioles with the production of a bronchiolitis, peribronchiolitis, and bronchopneumonia.

Protocol 2.—Monkey 142. *Cebus capucinus*, female; weight 1,300 gm. Aug. 26, 1919. Received 0.5 cc. of *B. influenzæ* peritoneal exudate in the nasal cavities. Aug. 29. Killed.

Anatomical Diagnosis.—Acute rhinitis; acute sinusitis of left antrum; bronchopneumonia, right lower lobe.

Autopsy.—Subject is a small Capuchin; weight 1,300 gm. Lungs show no definite consolidation in the gross. Turbinates greatly swollen and reddened and covered with mucus. Left antrum contains blood-tinged cloudy fluid. Right antrum is clear.

Cultures.—Left antrum, *Streptococcus viridans*; trachea, no growth.

Microscopic Examination.—Turbinates.—Extensive infiltration of leucocytes in mucosa and submucosa. *Right lower lobe.*—Shows an acute bronchiolitis with early pneumonic changes. At certain points the epithelium of the bronchiole is absent and its place taken by a deposit of fibrin and leucocytes. The wall of the bronchiole is infiltrated with polymorphonuclear leucocytes and lymphoid cells and the infiltration extends into the alveolar walls of the adjacent tissue. The capillaries of the alveolar walls are markedly distended in this neighborhood, and a few red blood cells and leucocytes have escaped into the alveoli. The changes, however, are for the most part interstitial and sharply limited to the vicinity of the bronchioles. Section of the right lower lobe stained for bacteria shows no influenza bacilli or other bacteria.

This case illustrates *Bacillus influenzæ* pneumonia in the very earliest stage and indicates that the process is essentially peribronchiolar; in other words, a true bronchopneumonia. Unfortunately, the pneumonia was so early that it was not recognized at autopsy, so no cultures were taken from the lungs. There is every reason to believe, however, that *Bacillus influenzæ* was the exciting agent.

The second case presented a more extensive *Bacillus influenzæ* pneumonia. In this animal the process was readily recognized during life and at autopsy. The lungs showed the hemorrhagic edema and type of consolidation so frequently seen in *Bacillus influenzæ* pneumonia in man.

Protocol 3.—Monkey 157. *Macacus syrichtus*, male; weight 5,200 gm. Aug. 26, 1919. Received 2 cc. of *B. influenzæ* peritoneal exudate intranasally; also received 1 cc. of same peritoneal exudate in the throat. Aug. 29. Killed.

Anatomical Diagnosis.—Acute catarrhal rhinitis following instillation of *B. influenzæ*; bilateral sinusitis of antra of Highmore; acute tracheobronchitis and bronchiolitis; hemorrhagic edema of lungs; bronchopneumonia; vesicular emphysema; bronchiectasis; hyperplasia of thymus and thoracic lymph glands.

Autopsy.—Subject is a large male *Macacus*. Thymus moderately enlarged. A few old adhesions in both pleural cavities. The greater part of the right upper lobe is intensely congested and dark red in color. Emphysematous lobules stand out prominently over the engorged area. A small portion of the upper posterior part of the right lower lobe shows a similar engorgement and emphysema. There is a small patch of engorgement at the root of the right middle lobe. The cut surface of the involved portion of the right lung is dark red and oozes a considerable quantity of bloody fluid. The bronchi are very conspicuous on the cut surface and appear to be much distended. The left upper lobe is covered with old fibrous tags. There is a large area of engorgement along the posterior border similar to that in the right upper lobe. The bronchial lymph glands are greatly swollen. The mucous membrane of the trachea and bronchi is congested and covered with blood-tinged mucus. Liver and kidneys show slight cloudy swelling. Other abdominal organs present nothing of importance. Testes normal. Rectus abdominis muscle appears normal. Nasal mucous membrane is reddened and covered with mucus. Turbinates, especially the left, are intensely swollen and engorged, and have a purplish tinge. Both antra contain pus.

Cultures.—Left turbinate, *B. influenzæ* and *Staphylococcus albus*; right antrum, *B. influenzæ* and Gram-negative coccus; trachea, no growth; both bronchi, pure culture of *B. influenzæ*; right upper lobe, pure culture of *B. influenzæ*; right lower lobe, pure culture of *B. influenzæ*; left upper lobe, six colonies of *Streptococcus viridans*.

Microscopic Examination.—Turbinates.—Marked swelling of the epithelial cells. There is a deposit of blood and mucus on the surface of the epithelium. A considerable number of leucocytes are found working their way between the epithelial cells and there is moderate infiltration of leucocytes in the submucosa. *Trachea.*—Deposit of mucus containing leucocytes on surface of the epithelium. Many goblet cells; a considerable number of polymorphonuclear leucocytes working their way out between the epithelial cells. *Right upper lobe.*—Shows pneumonic process in several phases. In some places there are marked edema and

engorgement (Fig. 4). In other places the alveoli are filled with serum and red blood corpuscles, while at still other points an exudate of leucocytes, fibrin, and desquamated epithelial cells fills the alveoli. The process is very irregular and peribronchiolar in distribution. There is a striking absence of the perivascular edema and infiltration of leucocytes so frequent in pneumococcus and streptococcus pneumonia. Another feature is the marked alveolar emphysema which is seen at many points (Figs. 4 and 5). The alveoli are much larger than normally and the alveolar walls are thin. In many places the great distention of the alveoli has resulted in rupture of the walls. The bronchioles contain blood and coagulated plasma and in some places a leucocytic exudate (Fig. 5). The walls of the bronchioles are infiltrated with leucocytes, and their epithelial lining has been destroyed in places. Some of the bronchi and bronchioles are greatly dilated (Fig. 6), and their epithelial cells show marked flattening. There are chronic inflammatory changes with organization about some of the blood vessels, but these appear to be the sequel of an old infection. There are a few minute intracellular Gram-negative bacilli, all in the leucocytes in the alveoli or bronchioles. *Right lower lobe.*—There is considerable interstitial infiltration of leucocytes in the neighborhood of the bronchioles, and in such areas the alveoli also contain exudate. Emphysema well marked. There are a few influenza bacilli in some of the alveoli. The bacteria are intracellular, having been phagocytosed by the leucocytes composing the alveolar exudate. *Left upper lobe.*—Considerable congestion, but no exudate. No influenza bacilli found. *Thymus gland.*—Well marked hyperplasia of the follicles. There is considerable infiltration of eosinophils, especially around the small central lymph channels. The larger lymphatic channels are greatly dilated, and are filled with coagulated plasma. *Spleen, testes, pancreas, and rectus abdominis muscle.*—Normal.

The third case of *Bacillus influenzæ* pneumonia following experimental influenza also presented characteristic changes.

Protocol 4.—Monkey 159. *Macacus syrichtus*, male; weight 5,430 gm. Aug 27, 1919. Received 1 cc. of blood broth culture of *B. influenzæ* in each nostril, and 1 cc. in the throat. Sept. 1. Killed.

Anatomical Diagnosis.—Acute rhinitis; acute tracheobronchitis; bilateral sinusitis of antra; hemorrhagic bronchopneumonia, left upper and middle lobes, and right upper lobe; lobar pneumonia, stage of resolution and organization, left lower lobe; hyperplasia of thymus and thoracic lymph glands.

Autopsy.—Subject is a large male *Macacus*. Thymus greatly enlarged, extending down to the middle of the pericardium. It is pinkish yellow and measures 3 inches in length. Pericardial sac normal; heart slightly dilated. The lungs are voluminous. The left upper and middle lobes show, extending outward from the hilum, deep red patches of engorgement and edematous consolidation. In these patches emphysematous air sacs are prominent. Cut surface shows irregular patches of consolidation, and exudes a large amount of bloody frothy

fluid. In the upper and posterior portion of the left lower lobe there is an area of moderately firm consolidation about 3 cm. in length. The cut surface of this area is pale yellowish gray, slightly moist, and has a translucent appearance (old organization). It is sharply defined from the unconsolidated portions of the lobe. The right upper lobe shows a few small patches similar to those in the left upper lobe. The remainder of the lungs is crepitant.

Cultures.—Trachea, *B. influenzæ* and a few Gram-negative micrococci and *Streptococcus viridans*; bronchi, *B. influenzæ* and Gram-negative micrococci; left antrum, *B. influenzæ* and *Streptococcus viridans*; right antrum, pure culture of *B. influenzæ*; left upper lobe, pure culture of *B. influenzæ*; left middle lobe, no growth; left lower lobe, pure culture of *B. influenzæ*.

Microscopic Examination.—Left upper lobe.—Extensive consolidation in one part of the section, which is apparently of some duration, as well advanced resolution and organization are present in many places. The exudate is found chiefly in the alveoli, and consists of lymphoid cells and polymorphonuclear leucocytes. Desquamated epithelial cells are also found in considerable numbers in the alveoli. Some of the alveoli contain plugs of organizing fibrin. There is a thick zone of lymphoid cells and connective tissue around the blood vessels. The lesions are those of a resolving lobar pneumonia. The whole lobe, however, is not implicated. Just beyond the consolidated area, the tissue shows engorgement and hemorrhage into the alveoli. A few influenza bacilli are seen in the bronchi. *Left middle lobe.*—Main bronchus filled with blood and leucocytes. In the neighborhood of the large vessels the lung tissue shows intense engorgement and hemorrhage with extravasation of red blood corpuscles into the alveoli and moderate edema. Pleura has been converted into a thick coat of organizing granulation tissue with a deposit of fibrin on the surface (result of a previous infection). Peripheral portion of section is free from consolidation. There are patches of compensatory emphysema in some places. There are large numbers of influenza bacilli in the bronchi, but none in the alveoli. *Left lower lobe.*—Sections show an extensive exudate in practically all the alveoli, resembling that seen in pneumococcus pneumonia. Resolution and organization are in process. Marked perivascular infiltration. Bronchi filled with pus. No bacteria found. *Trachea.*—Deposit of mucus on surface of mucous membrane; mucosa and submucosa infiltrated with leucocytes (Fig. 2). *Tracheal lymph gland.*—Hyperplasia of follicles and infiltration of considerable number of polymorphonuclear leucocytes in the sinuses. Some of the leucocytes are undergoing disintegration and phagocytosis by macrophage cells. *Turbinate.*—Mucosa for the most part intact, but the epithelial cells are swollen and filled with mucus. Heavy deposit of mucus and leucocytes on surface of mucosa. Leucocytes are seen working out between the epithelial cells, and the submucosa is densely infiltrated with polymorphonuclear leucocytes, lymphocytes, and plasma cells. Blood vessels distended with red blood corpuscles. *Thymus.*—Marked hyperplasia of the follicles, and distention of lymph channels which are filled with coagulated plasma. The small capillaries in the follicles are filled with leucocytes.

In Monkey 159 we have a further illustration of *Bacillus influenzæ* pneumonia, in this instance, however, apparently superimposed on an old unresolved pneumonia of the lobar type. Many of the alveoli contain only desquamated epithelial cells or plugs of fibrin, while others are filled with young connective tissue cells. Such areas resemble an organizing pneumococcus pneumonia. On the other hand, extensive areas of hemorrhage and edema occur, and in the latter places the picture is that of a *Bacillus influenzæ* infection.

These cases of *Bacillus influenzæ* pneumonia in monkeys present altogether a fairly complete reproduction of *Bacillus influenzæ* pneumonia as it occurs in man. Complete absence or scarcity of fibrin, hemorrhage and edema, bronchiolitis and peribronchiolar infiltration, discrete or confluent bronchopneumonia, and, finally, emphysema and bronchiectasis, are characteristic lesions which have been demonstrated in both man and monkey. In these three cases the infection originated in the nose and throat and extended down the bronchial tree to the terminal bronchioles, exciting in them a purulent bronchiolitis. Peribronchiolar infiltration with involvement of the adjacent alveoli followed (Fig. 5). The hemorrhage and edema in these animals probably represent a reaction on the part of the lung to absorbed toxic substances; the emphysema is partially compensatory, but for the most part is doubtless caused by the obstructing plugs of pus in the bronchioles.

Bacillus influenzæ Pneumonia in Monkeys Following Intratracheal Injection of Bacillus influenzæ.

Experimental *Bacillus influenzæ* pneumonia was produced by injecting virulent influenza bacilli directly into the trachea, as described in the preceding paper. In Table II the results of these experiments are catalogued. It will be seen that in one of the ten monkeys injected the results were entirely negative (Monkey 135). In two others a tracheobronchitis developed, but the autopsy showed no involvement of the lungs (Nos. 20 and 108). In the remaining seven a *Bacillus influenzæ* pneumonia was definitely established.

Lobes Involved.—The left lower lobe was involved in six cases, and the right lower lobe in six cases. The left upper lobe showed changes

in five of the monkeys, the right upper lobe in only four. All the lobes were affected in two cases (Nos. 124 and 136).

The influenza bacillus was recovered in pure culture from the lungs in two out of the seven cases (Nos. 124 and 138). It was recovered from the trachea or bronchus in three animals (Nos. 124, 136, and 138). It was also cultivated from the trachea in one of the monkeys that failed to develop pneumonia (No. 20). It was isolated from the heart's blood in only one case (No. 138) and that was in the monkey that developed pericarditis in connection with pneumonia.

As will be seen from Table II the lesion in the lungs is described in seven cases as a bronchopneumonia. The solid hepatized lung, so characteristic of pneumococcus infections, was never observed in these animals. The consolidation was distinctly patchy, though the patches were often confluent, especially around the hilum. The hemorrhage and edema frequently seen in *Bacillus influenzæ* pneumonia in man were present, but were usually less intense in the monkey. The picture in some of these cases was doubtless modified to some extent by the comparatively large amount of culture injected into the trachea. This conclusion is supported by the fact that Monkey 155, injected with 4 cc. of killed culture, also developed moderate bronchopneumonic lesions. Apparently the amount of toxic substance present in the culture was capable in itself of setting up a considerable cellular reaction. Monkey 136 received only 1 cc. of peritoneal exudate and developed a markedly hemorrhagic type of pneumonia, similar to that which developed spontaneously in some of the monkeys with experimental influenza.

On removing the sternum in these cases the lungs were invariably found lying free in the pleural cavities. Neither the fibrinous exudate of pneumococcus pneumonia nor the empyema of streptococcus pneumonia was to be seen. On removing the lungs irregular patches of increased resistance could be made out on palpation, most marked around the hilum and along the posterior border where the foci of consolidation usually coalesced to form larger patches. The surface of the lung over these patches was dark red, or even hemorrhagic, and sharply defined from the surrounding pink tissue (Figs. 7 and 8). On section the patches were dark red and rather gelatinous looking. Bloody fluid could be squeezed from the patches of consolidation.

TABLE II.

Monkeys That Received B. influenzæ Intratracheally.

Monkey No.	Date of injection.	Amount injected.	Date killed.	Pathological findings.	Autopsy cultures.
	1919		*1919*		
124	Aug. 11	5 plates (5 cc.).	Aug. 13	Confluent bronchopneumonia, all lobes; tracheobronchitis; hyperplasia of thymus and thoracic lymph glands.	H. B., n. g. R. L., pure B. I. Tr., " " Br., " "
20	" 14	5 (5 ").	" 20	Tracheobronchitis; hyperplasia of thymus and thoracic lymph glands.	H. B., n. g. Tr., pure B. I.
108	" 14	1 plate (1 ").	" 19	Tracheobronchitis; hyperplasia of thymus and thoracic lymph glands; necrosis of peribronchial lymph glands.	H. B., n. g. Tr., "
136	" 14	1 cc. of peritoneal exudate.	" 19	Hemorrhagic bronchopneumonia, all lobes; edema of lungs; tracheobronchitis; bronchiolitis; hyperplasia of thymus, spleen, thoracic and abdominal lymph glands.	H. B., n. g. L. L., " R. L., " Right br., pure B. I.
135	" 14	1 cc. of peritoneal exudate.	Not killed.		
137	" 19	4 plates (4 cc.).	Aug. 21	Confluent bronchopneumonia, R. U., R. L.; tracheobronchitis; hyperplasia of thymus.	H. B., n. g. R. U., " Tr., "

138	Aug. 19	4 plates (4 cc.).	Aug. 21	Confluent bronchopneumonia, R. L., L. U., L. L.; tracheobronchitis; pericarditis; hyperplasia of thymus and spleen.	L. L., pure B. I. Pericard., pure B. I. H. B., pure B. I. Tr., " "
141	" 22	1 plate (1 ").	" 29	Resolving bronchopneumonia, L. U., L. L., R. L.; hyperplasia of thoracic lymph glands; rhinitis; tracheobronchitis.	H. B., n. g. Tr., " Turb., Staph. A.
149	" 23	4 plates (4 ").	" 28	Resolving bronchopneumonia, L. U., L. L., R. U.; hyperplasia of thymus and thoracic lymph glands; bronchiolitis.	H. B., n. g. L. U., " L. L., " Tr., "
152	" 26	1 cc. of peritoneal exudate.	" 27	Bronchopneumonia, R. L., L. L.; tracheobronchitis; hyperplasia of thymus; mediastinitis.	H. B., " R. L., " L. L., " Tr., "
155	" 26	4 plates (4 cc.) of killed *B. influenzæ*.	" 28	Bronchopneumonia, R. L., L. L.; bronchiolitis.	R. L., " Tr., "

H. B., indicates heart's blood.

Areas of compensatory emphysema were frequently observed, especially toward the periphery of the lobe (Fig. 9). The dense new growth of connective tissue, often found in late influenza pneumonia in man, was not present, no doubt for the reason that the monkeys were killed comparatively early in the course of the infection. The trachea and bronchi contained mucopurulent material, in some cases blood-tinged. The tracheal and interbronchial lymph glands were considerably enlarged.

Microscopically, the changes observed were similar to those which have already been described above in connection with spontaneous *Bacillus influenzæ* pneumonia following an attack of experimental influenza. Sections from the lung show disseminated areas of infiltration which are often located about a bronchiole. The lumen of the bronchiole is filled with polymorphonuclear leucocytes and lymphoid cells, and sometimes red blood cells are mixed with the leucocytes (Figs. 10 and 11). The epithelial lining of the bronchioles is partially or perhaps completely destroyed and the underlying tissue is densely infiltrated with leucocytes (Fig. 11). This infiltration extends out into the adjacent interstitial tissue and the contiguous alveolar walls (Fig. 12) which show intense engorgement of the capillaries. The alveoli in this neighborhood do not escape, but contain red blood cells, leucocytes, and desquamated epithelial cells. At times the engorgement of the alveolar walls is so severe that large numbers of red blood cells extravasate into the alveolar lumen. These foci of exudation may coalesce to form larger areas, but the process never reaches the stage of universal consolidation as seen in pneumococcus infections. Vesicular emphysema is present, but is usually not so striking as that in man. The larger bronchi contain pus and the columnar epithelial cells take on the goblet form. Pus cells are found working their way out between the epithelial cells lining the bronchus, and the submucosa is infiltrated with polymorphonuclear leucocytes and lymphoid cells. The pleura shows little if any deposit of fibrin. There is a striking absence of perivascular infiltration of leucocytes and of the lymphatic involvement, which were a prominent feature in pneumococcus and streptococcus pneumonia.

The following case is an excellent example of the findings after experimental *Bacillus influenzæ* pneumonia.

Protocol 5.—Monkey 136. *Cebus capucinus*, male; weight 1,100 gm. Aug. 14, 1919. Received intratracheally 1 cc. of exudate from peritoneum of monkey with *B. influenzæ* peritonitis. Aug. 19. Killed by blow on head.

Anatomical Diagnosis.—Experimental *B. influenzæ* bronchopneumonia; hemorrhagic edema of lungs; acute tracheobronchitis; bronchiolitis; hyperplasia of the thymus gland; hyperplasia of spleen, thoracic, mesenteric, and retroperitoneal lymph glands.

Autopsy.—Performed Aug. 19. On removing the sternum the lungs are found lying free in the pleural cavities, which are free from adhesions and fluid. The pericardial sac is normal. The anterior surface of the pericardium is partially covered by a greatly enlarged thymus gland, which measures about 0.5 cm. in thickness (Fig. 8). The gland is somewhat triangular in shape with the apex of the triangle pointing upward and the base of the triangle lying transversely across the pericardium. Cut surface of the gland is pale pinkish yellow and homogeneous, with definite lobules. Heart not enlarged. Myocardium and valves normal. Right lung is voluminous. Surface of all three lobes is smooth and glistening and everywhere mottled with small bright red hemorrhagic areas lying close together, in some places confluent, in others separated by narrow gray zones which apparently mark the boundaries between the lobules (Figs. 7 and 8). This hemorrhagic condition is also marked at the base and over the anterior surface of the lobes. The lobes have a boggy consistence and contain considerably less air than a normal lung, but the tissue has not the firm resilient consistence offered by a pneumococcus consolidation. Cut surface of right lower lobe shows engorgement and marked hemorrhagic edema. In the upper and middle lobes the congestion is noticed at the root of the lung and gradually fades out toward the periphery. The upper and middle lobes are similar to the lower lobe but show less involvement. The left lung is similar to the right in respect to its surface. The lower lobe shows the same striking hemorrhagic appearance, and the upper and middle lobes are involved to a less extent. Cut surface of the left lung is similar in general to that of the right. Tracheal and interbronchial lymph glands are enlarged and intensely congested. Trachea and bronchi are filled with bloody frothy mucus. Spleen is definitely enlarged. Cut surface is dark red. Liver and kidneys show cloudy swelling. Suprarenals normal. The retroperitoneal and mesenteric lymph glands are considerably enlarged. Pancreas shows no noticeable changes.

Cultures.—Right lower lobe, no growth; left lower lobe, no growth; right bronchus, pure culture of *B. influenzæ*.

Microscopic Examination.—Right upper lobe.—Intense engorgement of all blood vessels and capillaries. In many places the alveolar walls are greatly thickened on account of the distention of capillaries with red blood cells. In lower half of section there is a small patch of consolidation where the alveoli and their walls are infiltrated with many polymorphonuclear leucocytes. In addition to leucocytes the alveoli contain desquamated epithelial cells and red blood corpuscles. The bronchioles contain pus, and the walls of the bronchioles are infiltrated with leucocytes. Section stained for bacteria shows no influenza

bacilli or other bacteria. *Left lower lobe.*—Section shows engorgement, hemorrhage, and, in some places, considerable emphysema (Fig. 9). Considerable number of red blood corpuscles found in the alveoli. Stained section shows no bacteria. No leucocytic exudate in the alveoli in this section. *Left middle lobe* (*Fig. 10*).—Shows marked engorgement and hemorrhage similar to that in the left lower lobe. *Thymus.*—The follicles are greatly enlarged, due to hyperplasia of the chyme centers and to the greatly increased number of lymphoid cells. Sinuses are distended, being packed with lymphoid cells. The fatty tissue between the follicles is compressed to a thin layer. No polymorphonuclear leucocytes or bacteria are seen. *Trachea.*—Shows no striking changes. The capillaries beneath the epithelium are engorged. The epithelial cells are swollen and filled with mucus. There is a thin layer of mucus on the epithelial surface in which a few red blood corpuscles and lymphocytes are seen. There is no hemorrhage or cellular infiltration in the submucosa. *Tracheal lymph node.*—Greatly enlarged. The vessels and capillaries are intensely engorged. The follicles show hyperplasia. The lymph channels are distended and filled with lymphoid cells and red blood corpuscles. Many of them contain coagulated plasma. In places there is considerable hemorrhage into the follicles. *Kidneys.*—Appear normal, except for moderate capillary engorgement. *Suprarenals.*—Normal. *Spleen.*—There is a very marked lymphoid hyperplasia, the follicles being greatly swollen, very prominent, and densely packed with lymphoid cells. There is slight congestion of the blood vessels and capillaries. The sinuses are filled with lymphoid cells.

Bacillus influenzæ Pericarditis.—One of the monkeys with experimental *Bacillus influenzæ* pneumonia developed an acute pericarditis (No. 138). Cultures from the pericardial fluid gave a pure growth of *Bacillus influenzæ*. The case is of such unusual interest that the autopsy findings will be given in detail.

Protocol 6.—Monkey 138. *Macacus syrichtus*, female; weight 2,915 gm. Aug. 19, 1919. Injected intratracheally with 4 cc. (four chocolate agar plates) of *B. influenzæ*. Aug. 21. Killed by blow on head.

Anatomical Diagnosis.—Experimental bronchopneumonia (*B. influenzæ*), left upper and lower and right lower lobes; acute serofibrinous pericarditis; chronic fibrous pleuritis, right side; acute tracheobronchitis; hyperplasia of thymus and spleen; focal necrosis in thymus; cloudy swelling of viscera.

Autopsy.—Performed Aug. 21. Subject is a medium sized female *Macacus*. On removing the sternum thymus gland is found considerably enlarged. There are a few adhesions in the right pleural cavity. Left pleural cavity normal. On opening the pericardium a moderate quantity of cloudy fluid escapes. Both the visceral and parietal layers of the pericardium are opaque and show marked edema, but there is very little fibrin. Heart moderately enlarged. Myocardium and valves normal. Left lung lies free in the pleural cavity. Anterior portions appear nor-

mal. Posteriorly, however, there are grayish red translucent patches of consolidation. These areas involve a considerable part of the lower lobe. In the upper lobe the process is less extensive. In both cases the changes appear to start at the hilum and spread outward. The right lung is bound to the chest wall by old fibrous adhesions. On section upper and middle lobes are normal. Lower lobe shows a small patch of pneumonia, similar to that found in the left lung. Tracheal and bronchial mucous membrane is congested and covered with mucus. Liver and kidneys show cloudy swelling. Other abdominal organs appear normal.

Cultures.—Left lower lobe, pure culture of *B. influenzæ*; pericardium, pure culture of *B. influenzæ*; heart's blood, pure culture of *B. influenzæ*; trachea, pure culture of *B. influenzæ*.

Microscopic Examination.—Trachea.—Mucus on epithelial surface. Moderate number of lymphoid cells and polymorphonuclear leucocytes in submucosa. Leucocytes also found between epithelial cells. Another section at bifurcation of trachea shows mucus and pus cells on the mucosa and infiltration similar to that in the other section. *Right lower lobe.*—Sections show extensive infiltration of leucocytes in the peribronchial tissue and in the adjacent alveoli. The exudate consists of polymorphonuclear leucocytes, lymphoid cells, and, in addition to these, the affected alveoli contain desquamated epithelium, and a slight amount of fibrin in some places. Bronchi and bronchioles filled with pus. Deposit of fibrin and leucocytes on the pleura. Perivascular infiltration is not observed. Section stained for bacteria shows many minute Gram-negative bacilli (*B. influenzæ*) in the alveolar exudate, mostly intracellular, being in the leucocytes. No influenza bacilli are found in the alveolar walls. Bronchioles are filled with pus, with considerable destruction of epithelium. Another section from right lower lobe shows interstitial pneumonia. *Left lower lobe.*—Shows infiltration similar to that on right side; small plugs of fibrin in some of the alveoli. Another section shows numerous alveoli filled with dense plugs of fibrin, in which is suspended a moderate number of disintegrating leucocytes. A moderate number of intracellular influenza bacilli is found. This lobe shows bronchopneumonia. A few influenza bacilli are found in the alveoli. *Thymus.*—Marked hyperplasia of the follicles and of the chyme centers. The lymph channels are greatly distended and are filled with coagulated plasma. The connective and adipose tissue surrounding the gland is infiltrated at many points with polymorphonuclear leucocytes. There is an area of necrosis of considerable extent at one point in the parenchyma. *Pericardium and heart muscle.*—Serosa of the pericardium is covered with a thick vascular membrane of granulation tissue on the surface of which there is a deposit of fresh fibrin and polymorphonuclear leucocytes. At some points the exudate is necrotic. There is moderate infiltration of leucocytes in the superficial portion of the myocardium. The muscle fibrillæ show cloudy swelling. *Liver.*—Hydropic degeneration about periphery of lobules. *Kidney.*—Shows definite cloudy swelling; glomeruli congested. *Spleen.*—Hyperplasia of follicles.

Other Pathological Changes Found in Association with Experimental Bacillus influenzæ Pneumonia.—Tracheobronchitis was noted in five out of the seven cases. It differed in no way from the tracheobronchitis already described in connection with experimental influenza. Hyperplasia of the thymus was observed in six of the seven cases, and was also present in the two monkeys (Nos. 20 and 108) which developed only tracheobronchitis following the intratracheal injection of influenza bacilli. The microscopic changes were identical with those already described under experimental influenza.

Enlargement of the tracheal and interbronchial lymph glands was usually noted, and in one case (No. 136) the various groups of abdominal lymph glands also shared in this hyperplasia. Monkey 108 showed focal necrosis in the bronchial lymph nodes, and in Monkey 152 there was an acute mediastinitis. There was hyperplasia of the spleen in two instances (Nos. 136 and 138).

Cloudy swelling of the liver was quite common, and similar changes, though usually less marked, were observed in the kidneys, adrenals, and heart muscle. The testicle showed no degenerative changes. No foci of necrosis were found in the rectus muscles.

Bacillus influenzæ Pneumonia in Man.

Microscopic sections from a number of cases of pure *Bacillus influenzæ* pneumonia in man[6] have been studied in connection with experimental *Bacillus influenzæ* pneumonia in monkeys. Photomicrographs from the lungs of two of the cases are shown in Figs. 13 and 14. A number of the lesions described by Wolbach are well displayed in these reproductions.

The first section (Fig. 13) is from a patient who died on the 13th day of the disease. At autopsy the lungs showed confluent bilateral bronchopneumonia and bronchiectasis. Microscopically, as the photograph shows, there are the characteristic changes in the bronchioles described above. The lumen is plugged with an exudate of leucocytes and red blood cells and the lining epithelium has been destroyed in part. The wall of the bronchiole and the adjacent structures are engorged with red blood cells and infiltrated with leucocytes.

[6] These sections were obtained through the kindness of Dr. S. B. Wolbach.

The alveoli contiguous to the bronchiole are filled with coagulated plasma in which are imbedded leucocytes, red blood cells, and desquamated epithelium. Alveoli further distant from the bronchiole contain coagulated plasma but are mostly free from exudate. The alveolar walls are everywhere engorged with red blood cells.

The second patient died of *Bacillus influenzæ* pneumonia on the 9th day of the disease. The autopsy showed confluent bronchopneumonia of both lungs and hemorrhagic myositis of the pectoral and rectus muscles. Microscopic sections of this case (Fig. 14) show severer lesions than those of the previous case. Engorgement, hemorrhage, and edema are present, but in addition there is an extensive emphysema. A moderate grade of leucocytic infiltration is also noted, many of the leucocytes being of the small mononuclear type. The peculiar hyaline membrane of fibrin described by Wolbach is found lining many of the distended alveoli.

The lesions found in these two cases of *Bacillus influenzæ* pneumonia in man are closely simulated by those observed in the lungs of Monkey 157, which, it will be recalled, developed an attack of *Bacillus influenzæ* pneumonia following experimental influenza.

DISCUSSION.

The pathological changes observed in experimental influenza in monkeys differ in no essential respect from those which occur in human influenza. In both instances the disease manifests itself as an acute catarrhal inflammation of the upper respiratory tract, extending usually from the nasal cavities down into the trachea and bronchi. In both diseases the accessory sinuses are often involved and a secondary bronchopneumonia is a frequent complication.

If, however, experimental influenza in monkeys resembles human influenza, the analogy between experimental *Bacillus influenzæ* pneumonia in monkeys and spontaneous *Bacillus influenzæ* pneumonia in man may be said to be still more striking. For in the case of *Bacillus influenzæ* pneumonia in man there are certain characteristics which differentiate it from the pneumonia excited by other bacteria, and render it readily recognizable by the pathologist. The studies of Pfeiffer, and more recently those of MacCallum and Wolbach, have

defined these peculiar lesions very clearly. The intense engorgement, hemorrhage, and edema, the purulent bronchiolitis, the scattered foci of peribronchiolar consolidation, and the ensuing emphysema and bronchiectasis have been emphasized by these writers as anatomical marks which would distinguish the infection from ordinary lobar and lobular pneumonia of pneumococcus and streptococcus origin.

In the foregoing experiments it has been shown that by the injection into monkeys of virulent influenza bacilli, a type of pneumonia can be induced that corresponds in most respects with that which results from *Bacillus influenzæ* infection of the lungs in man. There are the same characteristic changes in and about the bronchioles, the same hemorrhage and edema, the same focal consolidation, and the same tendency toward emphysema and bronchiectasis in the later stages.

Experimental *Bacillus influenzæ* pneumonia in monkeys differs in one respect from the spontaneous disease in man. The process in monkeys appears to be the expression of a milder infection than that in man. The engorgement and edema are usually less marked in monkeys and the leucocytic infiltration is more pronounced. The difference, however, might reasonably have been expected. The human cases occurred during a very severe epidemic in which *Bacillus influenzæ* had presumably acquired exceptional virulence. The strain which was employed for producing the disease experimentally had been cultivated for weeks on artificial media and it was only with considerable difficulty that even a moderate grade of virulence was conferred on it for monkeys. Moreover, in some of the animals the experimental lesions were induced by massive doses of bacteria which might have possessed enough toxicity to excite independently a certain amount of cellular reaction. That massive doses alone are capable of setting up considerable inflammatory reaction is evidenced by Monkey 155, which received 4 cc. of killed influenza bacilli intratracheally and developed a mild bronchopneumonia with some exudation into the alveoli. In this connection it is interesting to note that when *Bacillus influenzæ* pneumonia developed spontaneously in monkeys as a complication of experimental influenza, the pathological changes of *Bacillus influenzæ* pneumonia in man were even more closely simulated than in the animals in which the disease was produced experimentally by direct intratracheal injection of massive

amounts of culture. Monkey 157, for example, developed *Bacillus influenzæ* pneumonia subsequent to the inoculation of *Bacillus influenzæ* in the nose and throat. The autopsy showed in addition to the peribronchial changes, hemorrhage, edema, emphysema, and bronchiectasis. The most characteristic changes were also observed in the lungs of the monkeys that were inoculated intratracheally with small amounts of culture.

The pathogenesis of *Bacillus influenzæ* pneumonia is apparently different from that of pneumococcus and streptococcus pneumonia. In both the experimental and the spontaneous disease the infection seems to travel down the bronchial tree into the bronchioles and subsequently involves the neighboring alveoli by contiguity. In pneumococcus and streptococcus pneumonia the bacteria penetrate the bronchial mucous membrane almost at once and spread rapidly through the perivascular lymph spaces to the alveolar walls in all parts of the lobe. In *Bacillus influenzæ* pneumonia the bacteria are present in large numbers in the bronchioles and to a less extent in the adjacent alveoli. Influenza bacilli, however, are rarely found in any part of the interstitial tissue, and, as MacCallum has pointed out, the lymphatics are noticeably free from involvement in *Bacillus influenzæ* pneumonia. This may explain why the influenza bacillus rarely invades the blood stream. The influenza bacillus lacks the invasive character possessed by the pneumococcus and streptococcus; consequently the lesions produced by the first organism are focal, while those produced by the last two usually involve extensive areas. The influenza bacillus, growing, in a sense, outside the body, injures chiefly contiguous parts, probably by means of toxic substances which it secretes; the pneumococcus and streptococcus, invading the lymphatics, reach all parts of the lung and excite a massive lesion. MacCallum believes that suppurative bronchiolitis, with peribronchial pneumonia, may be caused in some instances by the streptococcus. There is always a possibility, however, that in such cases the influenza bacillus has been the first to infect the lung and has later been supplanted by the streptococcus as a secondary invader. In view of the experiments reported in this paper such an explanation seems fairly plausible. In a number of cases of experimental *Bacillus influenzæ* pneumonia sterile cultures were obtained from the lungs at autopsy,

but there was no question as to the etiology of the pneumonic process. The influenza bacillus had produced its characteristic inflammatory reaction and had then been overcome by the resistance of the animal.

CONCLUSIONS.

1. Virulent influenza bacilli, when injected into the nose and throat of monkeys (*Cebus capucinus* and *Macacus syrichtus*), excite an acute inflammation of the upper respiratory tract, characterized by swelling and hyperemia of the mucous membrane, infiltration of the mucosa and submucosa with leucocytes, desquamation of epithelial cells, and the production of a mucopurulent exudate. The accessory sinuses are often implicated in the infection.

2. Experimental *Bacillus influenzæ* infections of the upper respiratory tract are frequently accompanied or followed by bronchiolitis, peribronchial infiltration, and bronchopneumonia with hemorrhage and edema in the early stage, emphysema and bronchiectasis in the later stages. In general, the process closely resembles uncomplicated *Bacillus influenzæ* pneumonia in man.

3. The injection of virulent influenza bacilli directly into the trachea of monkeys induces in them an experimental bronchiolitis and hemorrhagic bronchopneumonia, similar in all respects to spontaneous *Bacillus influenzæ* pneumonia.

4. In experimental *Bacillus influenzæ* infections of either the upper or lower respiratory tract the influenza bacillus can usually be recovered during the acute stage by culture, either pure or in association with other bacteria.

5. In experimental *Bacillus influenzæ* infections in monkeys characteristic changes occur in the thymus gland—hyperplasia of the follicles, distention of the lymphatic channels, and infiltration of the parenchyma with leucocytes. This enlargement appears to be merely part of a general hyperplasia of the lymphoid structures in the cervical and thoracic regions.

EXPLANATION OF PLATES.

PLATE 90.

FIG. 1. Monkey 139. Experimental influenza. Section of a turbinate showing engorgement of capillaries in the submucosa, and profuse exudate of mucus and pus cells on the surface of the epithelium. × 100.

FIG. 2. Monkey 159. Experimental influenza followed by *B. influenzæ* pneumonia. Section from the trachea showing infiltration of leucocytes in the mucosa and submucosa. Deposit of mucus and leucocytes on the surface. × 400.

PLATE 91.

FIG. 3. Monkey 137. Experimental *B. influenzæ* pneumonia. Section of the trachea showing acute tracheitis. × 400.

FIG. 4. Monkey 157. Experimental influenza followed by *B. influenzæ* pneumonia. Section from the right upper lobe showing hemorrhage, engorgement, and emphysema. The alveoli contain fibrin and many red blood cells, also a few leucocytes and desquamated epithelial cells. × 100.

PLATE 92.

FIG. 5. Monkey 157. Experimental influenza followed by *B. influenzæ* pneumonia. Section from the right upper lobe showing bronchiolitis, peribronchial infiltration of leucocytes, hemorrhage, and edema. *Cf.* with Fig. 13. × 100.

FIG. 6. Monkey 157. Experimental influenza followed by *B. influenzæ* pneumonia. Section through the entire upper right lobe, showing peribronchial pneumonia, bronchiolitis, marked bronchiectasis, and emphysema. × 3.

PLATE 93.

FIG. 7. Monkey 136. Experimental *B. influenzæ* pneumonia. Hemorrhagic and edematous infiltration. This photograph illustrates the patchy character of the consolidation.

FIG. 8. Monkey 136. Anterior view showing hyperplasia of the thymus, which extends down over the greater part of the pericardial sac.

PLATE 94.

FIG. 9. Monkey 136. Experimental *B. influenzæ* pneumonia. Section through the entire left lower lobe showing hemorrhage, engorgement, and edema, with emphysema. × 3.

FIG. 10. Monkey 136. Experimental *B. influenzæ* pneumonia. Left middle lobe. Bronchiolitis, peribronchiolitis, engorgement, and hemorrhage. × 100.

PLATE 95.

FIG. 11. Monkey 124. Experimental *B. influenzæ* pneumonia. Section of the left lower lobe showing purulent bronchiolitis, partial destruction of the wall of a bronchiole, and peribronchiolar pneumonia. × 130.

PLATE 96.

FIG. 12. Monkey 152. Experimental *B. influenzæ* pneumonia. Section from the left lower lobe showing infiltration of leucocytes in the alveoli and septa adjacent to terminal bronchioles. × 100.

FIG. 13. *B. influenzæ* pneumonia in man (Dr. Wolbach's case). Acute bronchiolitis and peribronchiolar infiltration of leucocytes; engorgement and edema. *Cf.* with Fig. 5. × 100.

PLATE 97.

FIG. 14. *B. influenzæ* pneumonia in man (Dr. Wolbach's case). Extensive hemorrhage, engorgement, and edema; hyaline membrane lining alveoli; emphysema. × 130.

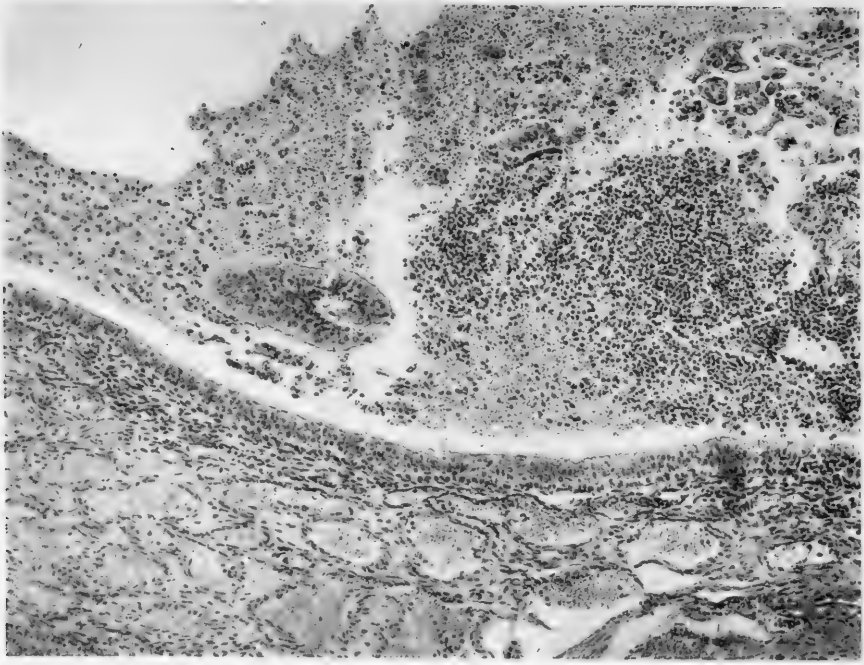

FIG. 1.

FIG. 2.

(Cecil and Blake: Experimental pneumonia. X.)

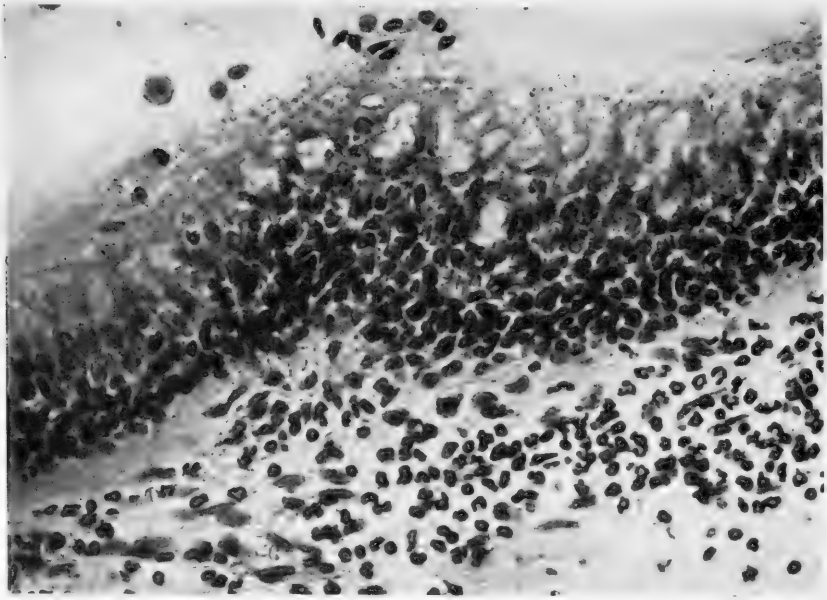

FIG. 3.

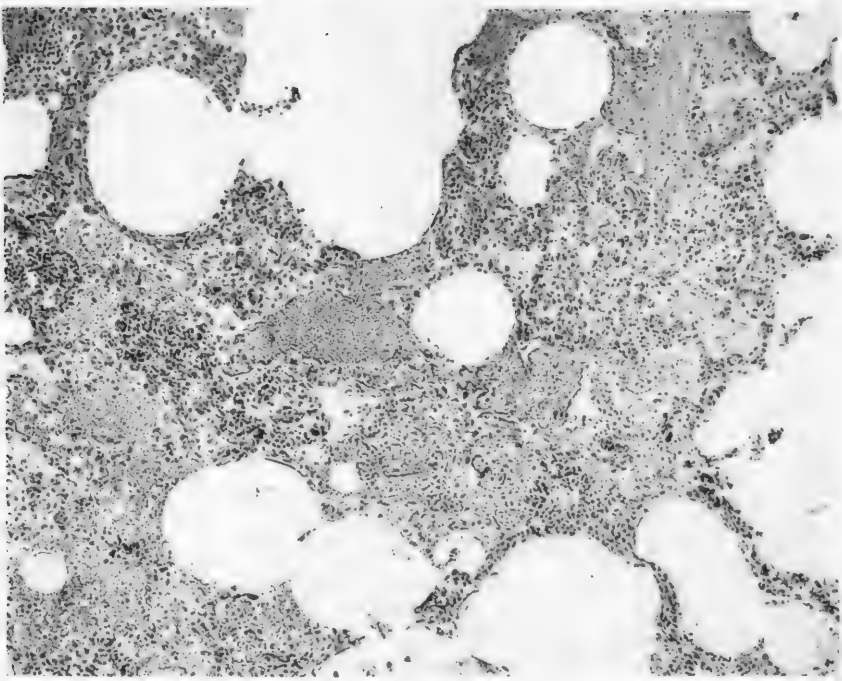

FIG. 4.

(Cecil and Blake: Experimental pneumonia. X.)

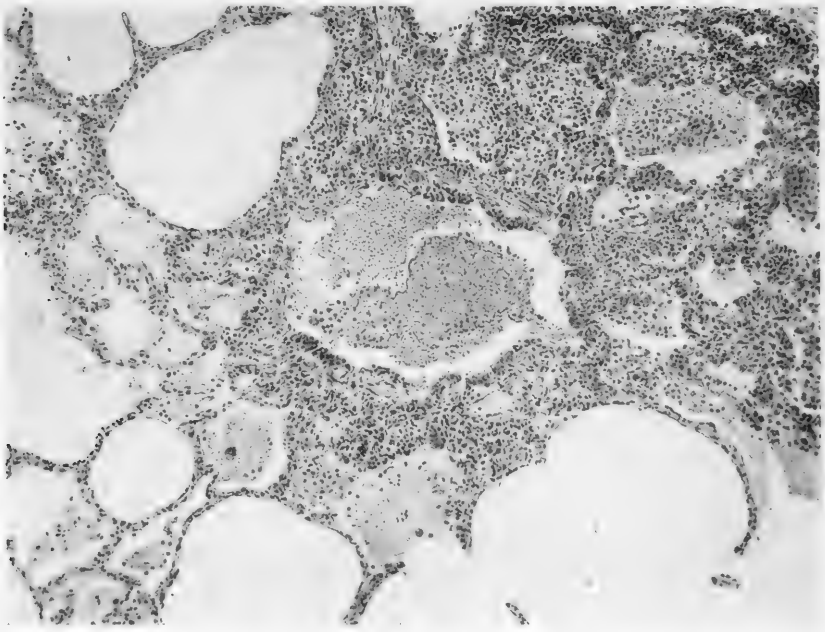

FIG. 5.

FIG. 6.

(Cecil and Blake: Experimental pneumonia. X.)

FIG. 8.

FIG. 7.

(Cecil and Blake: Experimental pneumonia. X.)

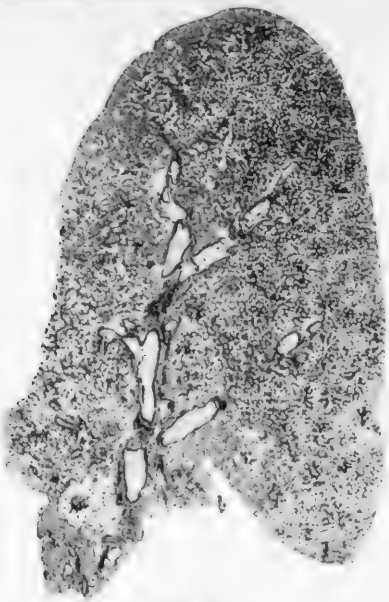

FIG. 9.

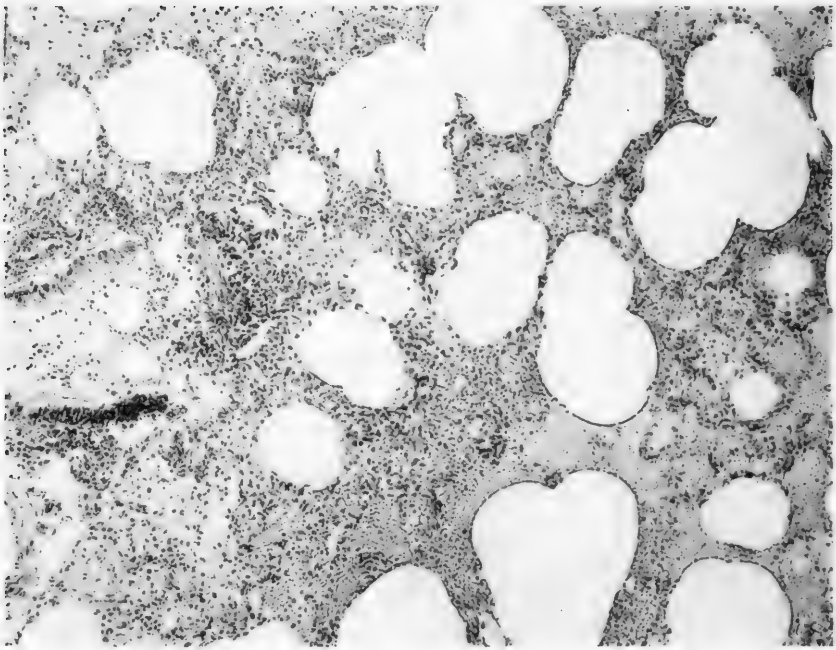

FIG. 10.

(Cecil and Blake: Experimental pneumonia. X.)

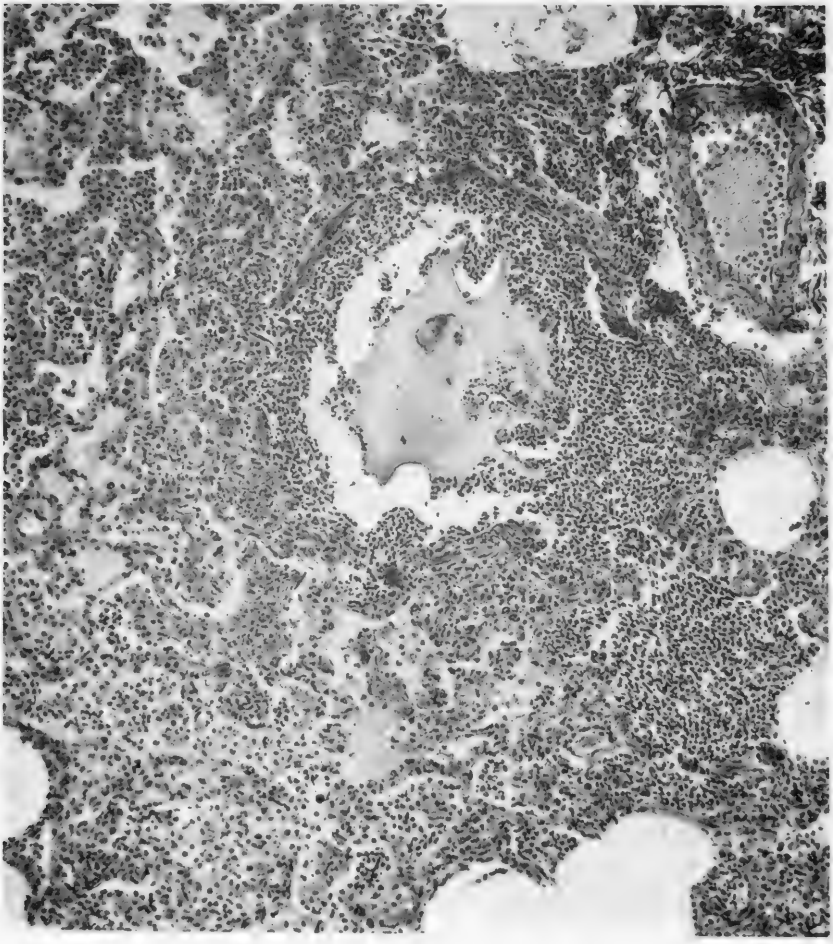

FIG. 11.

(Cecil and Blake: Experimental pneumonia. X.)

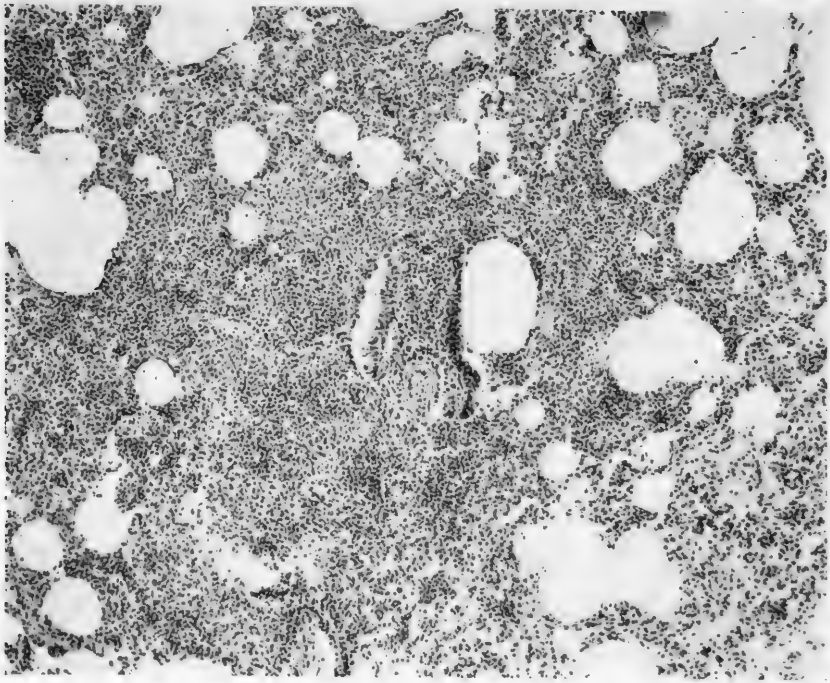

FIG. 12.

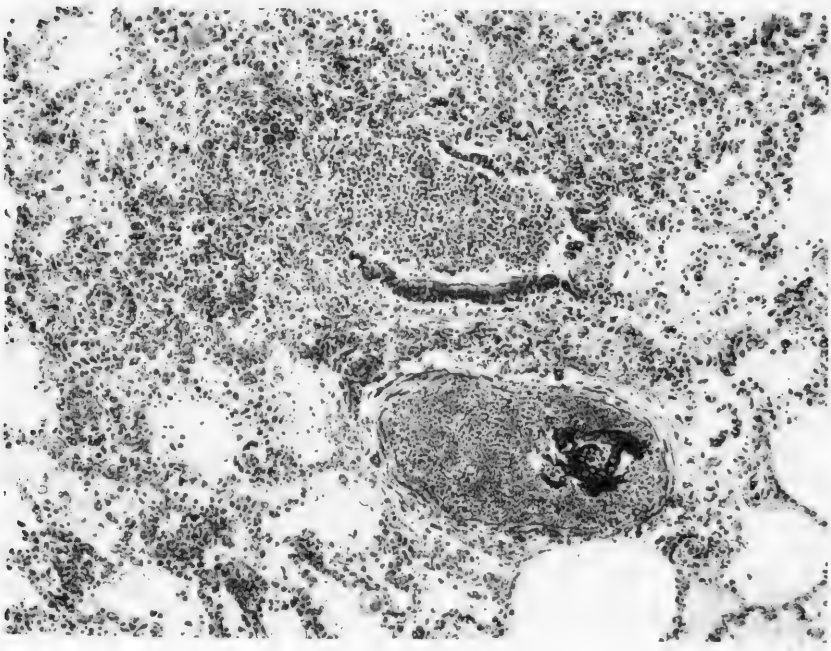

FIG. 13.

(Cecil and Blake: Experimental pneumonia. X.)

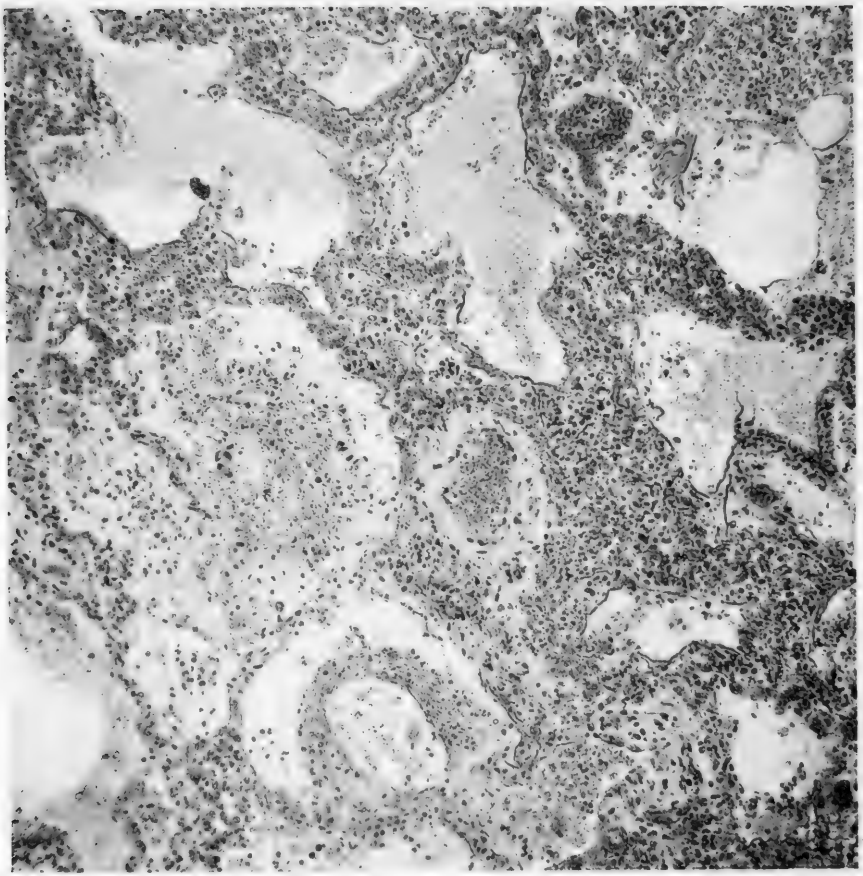

FIG. 14.

(Cecil and Blake: Experimental pneumonia. X.)

EXPERIMENTAL STUDIES ON HETEROPLASTIC BONE FORMATION.*

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Plates 98 to 100.

(Received for publication, June 22, 1920.)

The occurrence of bone in abnormal locations has been frequently reported in the literature. Its experimental production in various species of animals has also been recorded by a number of workers. Recently one of us (1) described a case of bone formation in the Fallopian tube and gave a discussion of the possible mode of its origin, based upon a review of the literature and a clinical and histological study of the case. At that time it was observed that most of the experimental work done was in connection with different problems, such as the origin of the blood cells and the restoration of renal function, and that for this reason reliable and convincing data on the mode of origin and growth of aberrant bone were not available. Though almost all investigators agreed that such a bone arises from fixed tissue cells by a process of metaplasia, there were great differences of opinion as to the factors initiating the process, and as to whether the proliferating cells become osteoblasts or merely a hyaline connective tissue which later is directly converted into osseous tissue.

The view credited to von Hansemann (2) that aberrant bone formation owes its origin to misplaced islands of perichondrium or cartilage was early rejected as insufficient to explain the frequent occurrence of bone in sclerotic vessels, in necrotic and calcified areas in the lungs, the gastric mucosa, and in other organs in no way associated in their development with cartilage. From a study of pathological specimens Pollack (3), Mönckeberg (4), Bunting (5), Buerger and Oppenheimer (6), and others (7) presented what they believed to be histological evidences of metaplasia. By experimental investigation Sacerdotti and Frattin

* Presented in abstract at the 69th meeting of the Washington University Medical Society, St. Louis, May 3, 1920.

(8), Liek (9), and Poscharissky (10) showed that bone can be produced in the rabbit kidney by ligating the vessels. Maximow (11) described various stages in this bone formation in the rabbit kidney in connection with his studies on the histogenesis of blood cells. Pearce (12) described similar bone formation in the dog kidney after removal of bits of its tissue, and concluded that the bone was produced by the direct ossification of scar tissue. In the aorta of the rabbit Harvey (13) was able to produce bone as well as cartilage by painting the external wall with a solution of silver nitrate in some cases and with one of cupric sulfate in others.

In every instance in which bone was produced in abnormal locations, there has been observed a proliferation of connective tissue cells and capillaries which seemed to migrate into the areas of necrosis and calcification. Bunting and others have favored the theory that the calcium salts act as a stimulus which induces the young connective tissue cells to differentiate into bone-forming cells. While the majority of writers on this subject thus believed the connective tissue cells to be chiefly concerned in bone formation, Thoma (14) was of the opinion that the osteogenic cells are derived from the endothelial cells of the capillaries that erode the calcific placques.

The rather frequent occurrence of cartilage in cases of heteroplastic bone formation as well as in Harvey's experiment suggested that possibly a primary conversion of connective tissue cells into cartilage may take place in some instances with subsequent formation of osseous tissue. Such a possibility was indicated also in the case of the Fallopian tube referred to above.

The bone marrow in aberrant bone was believed by Marchand (15) to arise from marrow cells transported through the blood stream, while Maximow was of the opinion that it was derived from the wandering cells or large lymphocytes, and Bunting thought it to be the result of a metaplasia of connective tissue cells.

It is thus apparent that although numerous observations both on human pathological specimens and animal experiments have been published, the opinions based upon them are conflicting. Owing to the accidental nature of the experimental investigations thus far carried out, detailed descriptions of the processes involved in the aberrant bone formation are not available. We have, therefore, decided to carry out further investigations as regards the mode of origin, growth, and detailed structures of the bone, as well as the probable part played by cartilage and calcium salts in initiating this bone formation.

Material and Methods.

Thirty-nine rabbits were divided into three series. In the first series of twenty animals renal vessels were ligated on one side.[1] In all but one animal the left side was chosen. In two of the animals the ureter was included in ligation and resection. In the second series of fourteen rabbits a portion of the ear and xiphoid cartilages was resected and transplanted into the subcutaneous tissue of the same animal. In a few instances the cartilages transplanted were previously boiled in water. The five animals comprising the third series were subjected to subcutaneous and intramuscular injections of calcium salts, consisting of calcium chloride and calcium carbonate, and of sodium phosphate.

All the animals were killed at various intervals in order to follow progressively the changes that were brought about by the experiments. The tissues were fixed in 4 per cent formaldehyde solution, decalcified in a mixture of phloroglucin and nitric acid, embedded in celloidin, and stained in hematoxylin and eosin. In some instances the Weigert-Van Gieson method of staining was resorted to in order to differentiate the tissues. Many of the kidneys were sectioned *in toto* for the purpose of studying different areas of the kidneys.

Series 1. Ligation of Renal Vessels.

Experimental Data.

Animal 1. 3 Days after Ligation.—The left kidney appears to be practically normal in size. It is dark red in color and soft in consistency and cuts easily throughout its entire extent.

Microscopic Examination.—The parenchyma of the kidney appears definitely hyaline with nuclei more pycnotic than normally. Hyaline casts occupy the lumina of the tubules. The capsule is edematous and infiltrated with lymphocytes, and presents a beginning fibroblastic proliferation. The transitional epithelium of the calices is desquamated *en bloc* at various places, and the cells are somewhat hyaline. Granular calcium deposits are noted along a layer of leucocytes which occur close under the capsule.

Animal 2. 7 Days after Ligation.—The left kidney is somewhat larger than the right and is very soft. The capsule is thickened and the cortex pale. There is a gritty sensation on cutting through the organ.

[1] All operations were performed under ether or urethane anesthesia.

Microscopic Examination.—Appearance similar to that in the previous animal except that the hyaline degeneration of the renal tissue is more pronounced and the epithelium of the calices appears healthy at places. Calcium deposits are noted under the capsule.

Animal 3. 14 Days after Ligation.—The left kidney is smaller than the right, soft to touch, and grayish in color. A slight gritty sensation is elicited upon cutting through the tissues.

Microscopic Examination.—Sections show an invasion of the hyaline cortex by fibroblasts from the capsule, accompanied by lymphocytes and capillary blood vessels. The granular calcium deposits have increased slightly in amount and are confined chiefly to the outer borders of the cortex.

Animal 4. 27 Days after Ligation.—The left kidney is markedly diminished in size and is completely surrounded by a mass of fatty tissue. The cortex is hard and cuts with difficulty. The ureter is grayish white and elastic. The pelvis of the kidney contains a soft, elastic, grayish white tissue mass.

Microscopic Examination.—An increased amount of calcium deposits is noted in the cortex and also a slight increase in the medulla. The cortex is invaded by a fresh granulation tissue with numerous lymphocytes.

Animal 5. 28 Days after Ligation.—The left kidney is slightly smaller than the right and appears grayish red. The capsule is surrounded by a mass of fat. The organ cuts with a distinctly gritty sensation. The region about the pelvis is filled with a grayish white, pliable mass of tissue. No bone can be made out on gross inspection.

Microscopic Examination.—Sections show calcific deposits in the cortex and in the medulla close to the calices. No noticeable amount of calcium is found in the medulla. The entire parenchyma is hyaline and necrotic. The cortex is infiltrated with lymphocytes, and is invaded by numerous fibroblasts and capillaries. Several multinuclear giant cells are found in this area. The calcification involves the interstitial tissue as well as tubular and glomerular epithelium and also the walls of the blood vessels. The calices are covered completely by healthy looking transitional epithelium which lines also many of the collecting tubules. Numerous calcific deposits are noticed in the calices and are surrounded by young connective tissue. Lymphocytic infiltration is well marked in this area. These calcific deposits are being invaded by fibroblasts and capillaries, and their edges have become irregular as if an erosion is taking place.

Surrounded by a mass of connective tissue in the proximity of the epithelium of the calyx there is a small spicule of bone whose periphery is lined by irregular layers of fibroblast-like cells, the axis of whose nuclei is chiefly perpendicular to the surface of the bone. Some of the bone cells along the edge of the spicule still retain the shape of the cells which cling to the periphery, while others have assumed an appearance typical of well developed bone corpuscles. No marrow cavity or Haversian system is as yet formed. There is, however, a small dent on the side of the bone in which are found a minute capillary containing a few erythrocytes, a row of fat spaces, and several fibroblasts evidently migrating

from the surrounding connective tissue areas. A multinuclear giant cell of the osteoclast type clings to the edge of the bone.

Animal 6. 32 Days after Ligation.—The macroscopic appearance of the left kidney resembles closely that of the previous animal. The cut surface, however, shows several small bony shells along the pelvis of the kidney.

Microscopic Examination.—Same general appearance of the cortex and medulla as that of the previous animal. The number of spicules of bone is now greatly increased. Lying underneath the epithelium of the calices and separated from it by connective tissue layers of variable thickness, these spicules are at several places directly continuous with a rather dense connective tissue. In most places this connective tissue is separated from the bone by a layer of cells not unlike periosteum, but in other places its fibers merge directly into the bone. If one traces such an osteoid tissue outward into the medulla, one finds that it is continuous with the connective tissue which extends from the capsular regions.

The spicules of bone are perforated by cavities of various sizes, and their edges are made irregular by numerous indentations and projections. These cavities and indentations are lined by a layer of cells closely resembling periosteum or endosteum. Some of these cells are intimately united through their cytoplasm with the matrix of the bone, as if they are being converted into bone corpuscles. Fibroblasts and capillaries occupy these cavities; a few leucocytes of mononuclear as well as polynuclear variety are also found outside the capillaries. Numerous clear spaces encircled by narrow rims of cytoplasm are seen between the fibroblasts, giving rise to an appearance similar to that of reticular connective tissue. Some of the fibroblasts seem to have lost their characteristic tapering protoplasmic processes and appear rounded off. A few multinuclear giant cells with oxyphilic cytoplasm are noted along the edge of the bone.

Animal 7. 33 Days after Ligation.—In this animal a segment of ureter just below the pelvis had been resected at the time of the operation. The gross appearance of the kidney is about the same as in the two preceding animals.

Microscopic Examination.—Practically the same appearance that was found in Animal 6, although the spicules of bone are fewer in number and the epithelium of the calices is thinner. The only two spicules of bone that are found lie under the epithelium and are directly continuous with the fibrous connective tissue which surrounds them. There is a close connection between the connective tissue growing in from the capsule and that proliferating in the calices.

Animal 8. 35 Days after Ligation.—The gross and microscopic pictures are practically identical with those described in the two preceding animals. Five or six centers of bone formation, lying under the epithelium, or around the buds of epithelium which are extending into the medulla, are noted. Most of them show a continuation from the connective tissue to bone as previously mentioned. Several spicules have a boundary layer of osteoblasts (Fig. 1). Cavities in the bone contain a fairly large number of myelocyte-like cells with finely granular, amphophilic cytoplasm and large single, vesicular nuclei of either oval or V shape. Mitosis is rather frequently encountered among the cells that occur within the

cavities of the bone. Coarsely granular eosinophilic forms of such cells also are found. Similar cells are met with in the connective tissue areas near the bone and also at a considerable distance from it. Fibroblasts and dilated capillaries also occur within the cavities. The connective tissue of the marrow now assumes a definitely reticular form.

Animal 9. 35 Days after Ligation.—The gross appearance of the left kidney is similar to that of the previous animal.

Microscopic Examination.—Sections show a great proliferation of buds of epithelial cells extending into the medulla of the kidney. Some of these buds form tubular structures with lumina in the center; others are so small and their epithelium is so flattened that they resemble capillary blood vessels with somewhat thickened intima; all such structures are, however, traceable by means of serial sections to the epithelial covering of the calices.

Spicules of bone are numerous and vary greatly in size. Where there is a dense connective tissue nearby a direct connection can be established between the bone and the connective tissue (Figs. 2 and 3). Many of the spicules lie in a highly cellular connective tissue; in such instances the bone is lined along the periphery by periosteal membrane. Cavities within the bone contain reticular connective tissue in which are suspended young fibroblasts, capillaries, and a few myelocyte-like cells elsewhere described. All the bone spicules lie close under the epithelium of the calices or around the epithelial buds. Degenerating epithelial cells with pycnotic nuclei and swollen granular cytoplasm, frequently containing clear, globular vacuoles, occur in the lumina of the pelvis and epithelial buds.

Animal 10. 35 Days after Ligation.—The left kidney is diminished in size and shows on gross inspection only a narrow layer of calcification under the capsule and a long shell of bone in the calyx.

Microscopic Examination.—The epithelial proliferation is similar in extent to that found in the previous animal. The calcium deposits are confined chiefly to the cortex, but are also found in the medulla. A few narrow but long spicules of bone are located in the proximity of the epithelium of the calices and around the buds of epithelium. They contain cavities which are similar in appearance to those found in the previous animal. The epithelium over the bone appears to be degenerated in many places.

Animal 11. 41 Days after Ligation.—The left kidney is small and shows on the cut surface a dense calcification in the cortex and small, hard foci of either calcification or ossification in the grayish medulla, close to the pelvis.

Microscopic Examination.—A considerable outgrowth of epithelial buds is found. Large spicules of bone are present in the connective tissue areas of the calices, a direct continuation between the bone and the connective tissue fibers being well demonstrated. The cavities in the bone contain the same structures as those found in the two preceding animals.

In areas close to the bone there is an areolar connective tissue which contains in its reticula a rather large number of mononuclear leucocytes of various

shapes and sizes. Most of these cells appear to be ordinary lymphocytes; others resemble myelocytes and possess a large cytoplasm, which takes up eosin stain, and oval or notched nuclei. Calcific placques are found in the neighborhood of the bone. The loose connective tissue described above is continuous with the connective tissue area surrounding the necrotic medulla of the kidney.

Animal 12. 54 Days after Ligation.—The left kidney is very much smaller than the right. It contains dense deposits of calcium throughout, and spicules of bone in the calices.

Microscopic Examination.—The entire renal tissue has been replaced by connective tissue, except in a small area where a trace of glomerular structure is still visible. Calcium deposit is noted practically everywhere in the organ, except in areas close to the pelvis where the connective tissue proliferation is most pronounced. Extension of epithelium of the calices into tubules has taken place, but the epithelial cells have become much lower, the nuclei mostly pycnotic, and the cytoplasm is vacuolated in many instances.

Spicules of bone are numerous and are more widely distributed than in any of the previous animals. In addition to those occurring near the pelvic epithelium there are several foci of ossification in the medulla. In the latter situations many small spicules are being laid down in such a way as to become directly continuous with calcific placques. These placques are surrounded by a large number of fibroblasts, capillaries, and comparatively few lymphocytes. A few fibroblasts are seen clinging to the edge of the calcium deposits, and become incorporated into their periphery, giving rise to an appearance similar to the new bone laid down along the lime plates (Fig. 5). In some instances epithelial buds derived from the calices are noted in the vicinity of these new forming bones; in other instances epithelial structures are nowhere to be seen. One of the bone spicules thus formed in direct contact with calcific placques contains in its cavities strings of fibroblasts, capillaries, and numerous leucocytes of various types. Some of these leucocytes are either lymphocytes or polynuclear neutrophils, but the majority resemble myelocytes. There are also quite numerous giant cells that cling to the edge of the bone. Megacaryocytes are also frequently seen, some of them being found in the act of phagocytosing myelocytes and polymorphonuclear leucocytes.

Near the calices the cavities occur within the substance of the bone or between it and the surrounding dense connective tissue. These cavities contain the same types of leucocytes that were described in the previous paragraph. It is among these myelocytes that mitotic figures are not infrequently observed. Some of the dividing myelocytes possess coarse, granular, eosinophilic cytoplasm, while others contain fine amphophilic granules.

Animal 13. 66 Days after Ligation.—The gross appearance of the left kidney is similar to that of the previous animal. Narrow shells of bone are visible close to the pelvis on the cut surface.

Microscopic Examination.—Sections show many narrow epithelial buds which have grown out into the medulla. A dense connective tissue extending from

the capsule meets that coming from the calices and together forms highly cellular layers around calcific deposits. Only three small spicules of bone are found immediately under the epithelium of the calices.

Animal 14. 71 Days after Ligation.—The left kidney is very much smaller than the right and forms a gray, stony, hard mass.

Microscopic Examination.—The medulla is found to be composed largely of organization tissue containing a great number of epithelial buds growing outwards from the calices. Numerous small spicules of bone are also found.

Animal 15. 94 Days after Ligation.—The left kidney is fairly large and cuts with considerable difficulty. It is completely surrounded by a mass of fat as in all other instances. The gross appearance of the cut surface does not differ materially from that in the previous animals, except that the bone spicules are distinctly larger, with specks of bright red color suggesting red marrow cavities.

Microscopic Examination.—Several large spicules of bone are found at the original site of the calices. Bone cells appear characteristic. Complete Haversian systems are quite numerous, the smaller ones containing only capillaries while the larger ones enclose, in addition, several cells closely resembling myelocytes. These Haversian canals are mostly lined by layers of cells which resemble endosteum. Lamellations of the bone are typical. Some of the Haversian canals may be traced into the larger cavities to be described immediately, and contain capillaries that are directly continuous with those found in the latter cavities.

The larger cavities above referred to lie either within the bone, between the bone and connective tissue, or within the wall composed of a number of smaller spicules of bone. Such cavities resemble bone marrow in several respects. Fat spaces are distributed in the cavities somewhat as in normal marrow, but seem to be less numerous. Between these fat spaces are found masses of leucocytic cells, the majority of which are mononuclear and transitional myelocytes of amphophilic variety. Eosinophilic and basophilic types also occur, but much less frequently. Numerous polymorphonuclear neutrophilic leucocytes and lymphocytes are also observed. Typical normoblasts and megaloblasts are present in small numbers.

There are from six to eight giant cells in each microscopic field, with ocular 4 and objective 3. Some of these possess single, deeply staining nuclei of large size while a smaller number are multinucleated. Several of these giant cells have evidently ingested polymorphonuclear leucocytes and myelocytes in various stages of degeneration (Fig. 4). Erythrocytes are scattered among the marrow cells as well as within capillaries. Mitotic figures occur not infrequently among the myelocytes.

Animal 16. 100 Days after Ligation.—The left kidney is small and is densely infiltrated with calcium deposit throughout, except at the pelvis where there is a soft, grayish white tissue mass. Bright red streaks about 3 mm. wide are noted at the corners of the pelvis and appear to be enclosed within thin shells of bone.

Microscopic Examination.—The cortex consists of masses of calcified tubules with hyaline connective tissue between them. The region of the pelvis contains

also hyaline connective tissue with numerous small round cells and fibroblasts. Near the calices which are lined by thin epithelium there are large numbers of tubules lined by an extension of the epithelium. There are large numbers of bone spicules in the calices, some of them extending so far out that they become directly continuous with calcified tubules of the medulla. In places they are pierced by epithelial tubules created by an extension of the epithelium of the pelvis. The bone cells are identical with those found in normal bone. In the spicules which are lying closer to the marrow cavities, however, some of the bone cells have become indistinct, the osseous tissue forming a deeply eosin-staining mass devoid of definite lamellæ and cell membranes. At several places the osseous tissue passes over gradually into hyaline connective tissue which contains also some young fibroblasts.

The marrow cavities contain the same type of leucocytic cells that were described in the previous animal. Myelocytes, megacaryocytes phagocytosing myelocytes and leucocytes, fat spaces, capillaries, and other constituents of the normal marrow are all present.

Such structures occur also in the connective tissue areas of the medulla and between the bone and the connective tissue. Wherever these structures are found some fragments of bone are usually present.

Animal 17. 104 Days after Ligation.—The gross appearance of the left kidney is similar to that of the previous animal. In the calices are noted a few grayish white shells of bone enclosing red streaks about 3 mm. wide and 1 cm. long.

Microscopic Examination.—The appearance is much the same as that previously described. In this kidney, however, the spicules of bone are slightly more numerous. The cavities that occur within the bone and between the bone and connective tissue resemble closely the normal marrow of the rabbit. Giant cells of megacaryocyte type are fairly numerous and are actively engaged in phagocytosis of myelocytes and polymorphonuclear leucocytes. One or two small sequestra without bone cells or endosteum are found lying among the mass of myelocytes. The edges of the bone facing the marrow cavites are markedly irregular and appear to be eroded, in these places the bone cells being indistinct and the endosteum absent. Masses of leucocytes may be seen piling up along the eroded edges.

Animals 18, 19, and 20.—The left kidneys in these animals are covered by a dense mass of fat. Upon opening this fatty capsule a collection of thick, creamy pus is found exuding from the very small kidney whose tissues have become extremely friable. Neither calcium nor bone is found.

Summary of Histological Findings.

Of twenty rabbits whose renal artery and vein were ligated, three became infected; of the remaining seventeen animals, bone was produced in all but the first four of the series. The presence of bone

became macroscopically evident as early as 32 days after ligation, and the bone continued to become larger and thicker until about the 54th day, when definite red marrow could be recognized. The sequence of events as we have observed it may be summarized briefly as follows:

Hyaline Degeneration.—3 days after ligation the kidney is swollen and dark red. Sections through the organ show a definite hyaline change in the cytoplasm of the renal tissues. The nuclei of the cells are more pycnotic than normally. No caryorrhexis has yet occurred, and the cells although hyaline are not yet completely necrotic. There is a zone of leucocytes close under the capsule. The epithelial covering of the calices is also hyaline and in places is desquamated *en bloc*. The progress of the degeneration of parenchymatous tissue, however, is slow, viable looking epithelial areas being present as late as 70 days after ligation.

Calcification.—A histologically recognizable degree of calcium deposition is observed 3 days after ligation. At this stage, however, it is present only as extremely minute traces of granular deposits and is confined to the tubular and glomerular epithelium along the zone of leucocytic infiltration under the capsule. 14 days after ligation the amount of calcium deposited is only slightly increased over that of the 3rd and 7th day stages and is still confined to the external portions of the cortex. 27 days after ligation the amount of calcification is decidedly increased and the process involves the medulla as well as the cortex. From this stage on until about 41 days after ligation the increase in the amount of calcium, as it is roughly estimated by histological appearance, is rapid and progressive, involving not only the epithelial elements but the interstitial tissue and the wall of the vessels as well. As the calcium salts are continually deposited, relatively large placques replace the formerly granular deposits. These placques are found in various shapes and sizes and occur in the cortex, medulla, and in the areas close to the pelvis. Quite frequently they present irregular indentations along their edges in which are found capillaries and fibroblasts migrating from the surrounding granulating tissue. Multinucleated giant cells also occur frequently in the neighborhood of these lime plates. New connective tissue grows in both from the capsule and from the calices and slowly replaces the necrotic tubules

and glomeruli, and surrounds completely the islands of calcium deposits. No fatty degeneration or infiltration has been noticed prior to calcification. The preliminary step to calcification as we have observed it consists thus of coagulation necrosis following the induction of anemia.

Ossification.—The first evidence of bone formation is noticed 28 days after ligation. From this time on, all the kidneys examined show the presence of more or less bone except in the three animals whose left kidneys had become infected.

The site of the occurrence of bone is consistently the connective tissue areas of the calices close under the transitional epithelium which is similar in appearance to ureteral epithelium. In earlier stages no other areas contain bone, whereas spicules are found well out in the medulla in some of the later stages. In no case, however, is there an evidence of ossification originating under the capsule. The total amount of osseous tissue increases on the whole, and the appearance of the bone becomes more characteristic as time goes on.

As early as 32 days after ligation thin bony shells are visible to the naked eye. By 94 days it is possible to foretell the presence of typical red marrow by observing with the naked eye streaks of bright red areas enclosed by spicules of bone. From 66 days on it becomes gradually more evident that the resorption of bone is more rapid than its formation. Multinucleated giant cells of the type usually spoken of as osteoclasts are more frequently seen in the later stages than in the earlier. These giant cells are closely applied to the periphery of the bone into which they appear to have penetrated to variable distances. At 94 days and thereafter sequestra make their appearance in the marrow cavities; some of the bone spicules become fragmentary and their bone cells less distinct than in the earlier stages; and in many instances the periphery of the spicules is much more irregular than formerly.

The processes of bone formation in the kidneys as we have observed them are as follows: Following the hyaline degeneration and calcification of renal tissues the connective tissue cells take part in bone formation in three different ways. In the first place, rather loose fibrous connective tissue is formed by a proliferation of fibroblasts that accompany the capillaries. The transitional epithelium of the

upper part of the ureter comes up with these structures. In the connective tissue thus formed under the epithelium a small spicule of bone makes its appearance. In general, the axis of bone cells is parallel with that of the surrounding connective tissue cells. An irregular layer of fibroblast-like cells surrounds the bone in a manner similar to the periosteal membrane; some of these cells have become continuous through their cytoplasm with the matrix of the bone, the nuclei still retaining their large vesicular form. A few lymphocytes as well as several loose fibroblasts lie close to the periosteum-like membrane (Fig. 1).

In the second type of ossification, seen frequently but only where the type described above has initiated the process and is still actively at work, there is a direct ossification of the hyaline connective tissue in continuity with the periphery of the preexisting bone (Figs. 2 and 3).

In the third type of ossification, seen only in one specimen in which epithelial buds and bone spicules extend far out into the medulla, the bone is formed directly in contact with the eroded edges of the lime placques. In this case the fibroblasts that erode their way with the capillaries into the calcified tubules seem to take on an osteogenic function and bone is laid down, largely in concentric lamellæ, against the lime deposits. In some places the bone formation begins at the periphery and surrounds a placque. As these various foci of ossification grow they produce coalescing conglomerates of true bone and calcium precipitate, which form large dense spicules, in which very little marrow is seen, though the Haversian canals contain highly vascular reticulum (Fig. 5).

Formation of Bone Marrow.—A slight suggestion of a cavity in the bone is present in the earliest stage at which the bone is found. This consists of a dent in the periphery of the spicule and is occupied by a row of a few fat spaces along the edge of the bone, fibroblasts migrating from the surrounding connective tissue areas, and a minute capillary containing a few erythrocytes. Within this cavity is seen a multinuclear giant cell of the so called osteoclast type clinging to the edge of the bone. Complete marrow cavities may be produced either through an enlargement of a dent in the periphery of the bone together with an ossification of the neighboring connective tissue areas, or

through bone formation around an area of reticular connective tissue elsewhere described. In earlier stages the contents of these cavities consist of fibroblasts, capillaries, and fat spaces. Leucocytes, chiefly small lymphocytes, are also found in them.

At the 35 day stage rather large numbers of myelocyte-like cells are noted. These cells possess a large cytoplasm with fine amphophilic granules and a large, vesicular, single nucleus of oval or V shape. Some containing coarse, eosinophilic granules also occur, but much less frequently. Such cells are found in groups quite apart from bone as well as within the marrow cavities. Whereas in the areas free from bone no mitotic figures can be observed, they are frequently seen among the cells lying within the marrow cavities. Direct evidences of genetic relation between these myelocyte-like cells and fibroblasts or large mononuclear leucocytes cannot be established in our series. Even when they occur away from the existing bone, such areas may later be surrounded by new forming bone. The typical marrow previously described is seen in the bones which are at least 5 weeks old. In the kidneys which contain such marrow, myelocytes are confined strictly to the cavities within the bone or to the close neighborhood of the bone. The mitosis of the myelocytes which was described for the 35 day stage is present even as late as 104 days after ligation. Apparently the abundance of myelocytes in the later stages owes its origin to a rapid proliferation of these cells.

Series 2. Autotransplantation of Cartilages.

In this series fourteen animals were killed from 7 to 80 days after subcutaneous autotransplantation of ear and xiphoid cartilage.

Experimental Data.

Animal 1. 7 Days after Transplantation of Boiled Cartilages.—Both xiphoid and ear cartilages are encapsulated in a very vascular, loose connective tissue.

Microscopic Examination.—The lacunæ in the pale blue matrix contain groups of hyaline cells with pycnotic nuclei. The perichondrium is stained deep red and the nuclei of the cells appear more pycnotic than normally; the inner and outer borders of the perichondrium are sharply defined. No indication of new formation of cartilage is found. The surrounding connective tissue is loose, vascular, and contains large numbers of leucocytes, both polymorphonuclear and small mononuclear.

Animal 2. 10 Days after Transplantation.—The gross appearance of the cartilages is similar to that in the previous animal.

Microscopic Examination.—The matrix of the cartilages is stained deeply with eosin; the cells in the lacunæ are normal in several places, their nuclei somewhat pycnotic elsewhere. The dense perichondrium is infiltrated to some extent with polymorphonuclear leucocytes and to a greater extent with small mononuclear leucocytes, and shows transitional stages of new cartilage formation. In places there are seen stellate groups of eight or ten cartilage cells in a homogeneous matrix deeply stained with hematoxylin. The loose connective tissue surrounding the transplants is richly cellular and is rather densely infiltrated with leucocytes.

Animal 3. 16 Days after Transplantation. Microscopic Examination.—The perichondrium and the surrounding connective tissue appear as in Animal 2, except that the infiltrating leucocytes are more predominantly mononuclear in type. The central part of the cartilage is stained deep red, the nuclei of the cartilage cells are more pycnotic, and their cytoplasm contains vacuoles. On the peripheral layers of the cartilages, especially of the ear cartilage, there are found several layers of newly formed cartilage cells.

Animal 4. 21 Days after Transplantation.—No changes in appearance are noted from that in the preceding animals, except that the leucocytic infiltration is less intense.

Animal 5. 28 Days after Transplantation.—The gross appearance of the transplants is the same as in the previous animals.

Microscopic Examination.—The lymphocytes infiltrating the surrounding connective tissue are few in number. There are several islands of newly formed cartilage separated from the original transplant of the ear cartilage by a small number of layers of perichondrial cells. Similar proliferation does not occur in the xiphoid cartilage.

Animal 6. 43 Days after Transplantation.—Both cartilages form hard masses in a fibrous capsule which is denser but less vascular than that seen in the previous stages.

Microscopic Examination.—Sections of the xiphoid cartilage show nothing unusual, the appearance being similar to that seen in the 1 week stage. The original cartilage appears as a rather faintly stained pink cast with empty lacunæ in many places. Lying on each side of it is a thick growth of new cartilage; that nearest the old cartilage consists of masses of deep blue cartilage cells with dark granules marking the nuclear structures, and separated from each other by a deeply red hyaline substance. Along the borders and out in the perichondrium are layers and islands of young, growing cartilage with a pale blue matrix and lacunæ containing cells with red, finely granular cytoplasm and nuclei which are either vesicular or, as in several places, somewhat pycnotic. The perichondrium merges into the surrounding dense connective tissue.

Animal 7. 59 Days after Transplantation of Boiled Cartilages. Microscopic Examination.—Sections show that most of the cartilages are represented by mere pink shadows with empty lacunæ, although in places there are cells which look

exactly like those in the 7 day stage, suggesting that either the boiling has not been sufficiently long or the cartilage cells have been fixed by being boiled. No newly formed cartilage is, however, noted in the perichondrial connective tissue. In places the lacunæ present a dark-blue-stained lining as though calcification were beginning to take place. In the dense connective tissue surrounding the cartilage a spicule of bone about 0.5 by 1 mm. in size is found. It contains a cavity filled with myelocytes, a few multinuclear giant cells, fat spaces, and reticular connective tissue. Its inner border is lined with a membrane of cells similar to endosteum (Fig. 6). Although it is difficult to rule out a possibility of its connection with cartilage, in all of half a dozen sections taken at various levels we are unable to establish such a connection.

The connective tissue surrounding the bone is rich in young fibroblasts, and the transition from osseous to connective tissue is gradual at one end. The bone cells are laid down in concentric circles around the marrow cavity and the fibroblasts around the bone are also so arranged; thus the bone and the immediately surrounding connective tissue form an eddy-like structure in a mass of fibrous tissue whose fibers run in lines parallel with the axis of the cartilage.

Animal 8. 67 Days after Transplantation. Microscopic Examination.—The sections show a condition about the same as that of Animal 4, except that there is more newly formed cartilage. Careful search shows no signs of ossification.

Animal 9. 69 Days after Transplantation. Microscopic Examination.—The xiphoid cartilage shows no well marked changes. The ear cartilage appears much like that seen in Animal 5. The original transplant appears totally necrotic, although in some places proliferating cartilage can still be seen on one side under the perichondrium. On the border of the dead cartilage there is a layer of pale blue, finely striated hyaline tissue which corresponds to the original perichondrium. Peripheral to this is a very thick layer of new cartilage. In two places bony spicules, 1 to 2.5 mm. in length, extend into the new cartilage from the newly formed perichondrium (Fig. 7). They seem to erode the cartilage and extend as far as the old perichondrium. They contain Haversian systems, marrow cavities, an endosteum-like membrane of cells, and in places incorporate into their substance shadows of cartilage cells.

Animals 10, 11, and 12. 72 Days after Transplantation. Microscopic Examination.—General appearance of sections is much like that found in the previous animals. In Animals 11 and 12 the old cartilage is fairly well preserved, and there is somewhat less growth of new cartilage than in Animal 8. All the newly formed cartilages are found in the form of islets of cells in the perichondrium.

Animals 10 and 11 show two, and Animal 12 shows one spicule of bone. All these bones are located in the perichondrium, and extend from it into the newly formed cartilage, exactly as in normal endochondral ossification. These bony spicules contain typical Haversian systems and are lined along their inner borders by an endosteum-like membrane (Fig. 8).

Animals 13 and 14. 80 Days after Transplantation.—Both specimens are similar to the previous ones in their gross appearance. Like them neither shows any marked changes in the xiphoid cartilage.

Microscopic Examination.—In both instances the original transplants of ear cartilage show definite evidences of degeneration, and there is definite growth of new cartilage from perichondrium. Animal 13 shows a round spicule of bone towards one end of the cartilage. The relation of connective tissue to bone is similar to that found in Animal 7.

Animal 14 shows a round spicule of bone near the ear cartilage transplant larger than the one just described. This spicule is surrounded by a thin layer of connective tissue on three sides so that it has the appearance of projecting from the perichondrium into an area of areolar connective tissue.

Summary of Histological Findings.

In the fourteen animals in which the ear and xiphoid cartilages were autotransplanted, bone was found in seven specimens of ear cartilages removed from 59 to 80 days after operation. One of these ear cartilages had been boiled previous to implantation. In xiphoid cartilages transplanted and removed under the same conditions and in the same animals as corresponding ear cartilages, bone formation did not take place.

Reaction of Tissue around the Transplant.—In the connective tissue around live or boiled transplants a definite inflammatory reaction is seen in the 7 day stage; polymorphonuclear and lymphocytic infiltration, an increase in the capillaries, and a proliferation of fibrous connective tissue cells are evident. This inflammatory reaction subsides gradually as seen in the 16, 21, and 28 day stages. The connective tissue surrounding the transplants blends completely with the perichondrium by the 43rd day, and makes it appear possible that it has taken up the function of perichondrium.

Changes Occurring in the Cartilage.—In the early stages the gradual degeneration and necrosis of the original cartilages can be followed. Beginning at the center the matrix assumes an eosin tint and the nuclei become pycnotic; the peripheral layers of the cartilage and the perichondrium appear healthy at earlier stages. The specimen of boiled cartilage removed in earlier stages shows practically the same general picture, except that here the perichondrium is entirely necrotic and only the matrix of the cartilage appears normal; the cells of the periphery retain their form and the nuclei stain deeply in hematoxylin. The general impression gained from such a picture is that the boiling may perhaps have fixed the cells. With the ear cartilage the periph-

eral layers seem to be actively proliferating, the new cartilage cells being formed from the perichondrium. In the case of the xiphoid cartilage, on the other hand, no change is seen after this time, the central portions remaining eosinophilic; the peripheral layers and the perichondrium are alive in many places, but no new cartilage is formed even in the later stages. In the ear cartilage the proliferation of new cartilage becomes evident as early as the 16 day stage and it increases as time goes on, although the proliferation is more pronounced with large than with small transplants. In some of the specimens of the ear cartilage removed from the 43 to 69 day stages in which large pieces of cartilage folded upon themselves had been used, the folds of old cartilage now entirely necrotic with empty lacunæ and faintly pink matrix can be traced through the whole section. On each side of this necrotic cartilage, and separated from it in some places by a layer of pale blue hyaline tissue representing the original perichondrium, are seen very thick layers of new cartilage with pink cytoplasm and nuclei varying in appearance from vesicular to pycnotic. The tissue adjacent to the healthy perichondrium resembles normal hyaline cartilage, but in the deeper layers the lacunæ are large and distorted and the matrix is stained deep blue. The general picture almost resembles that of a rapidly proliferating chondroma with the centers necrotic and calcified.

Ossification.—The earliest stage at which bone is found is the 59 day stage in the animal which received an implantation of boiled cartilages. In this animal there is very little evidence of new cartilage formation; the old cartilage appears dead in most places, although areas are found which suggest that possibly some of the peripheral cartilage cells are still alive, and in these areas a dense connective tissue which resembles perichondrium has been formed close around the cartilage. The spicule of bone consists of concentric lamellæ about an endosteum-lined cavity containing a typical marrow. The spicule itself is surrounded by a dense connective tissue whose fibers also run in concentric rings (Fig. 6). In places the bone and the connective tissue merge gradually into each other. A direct connection of the bone and the cartilage cannot be found in this specimen, but this fact does not signify the independence of the bone of any association with the cartilage, since our serial sections have not been complete.

Of the six animals with live cartilage transplants examined from the 69 day stage on, bone is found in all. In the two animals in which only a moderate amount of perichondrial cartilage formation occurred, the spicules of bone are similar in appearance and location to the spicule just described, although both are larger and contain less marrow. In the four animals in which an extensive new formation of cartilage took place with subsequent atrophic changes and calcification as previously described, from one to two bony spicules are found in each specimen, laid down immediately under the perichondrium and invading the calcified new cartilage. In no instance does the ossification occur in the original transplant, nor do the cartilage cells play an active part in the process, although lacunæ may be incorporated in the line of advancing bone formation. The cells of the perichondrium may be so closely associated with the bone cells that a distinction between the two often becomes difficult. Here, as in other instances, the fibers of the connective tissue about the bone are arranged like whorls around the peripheral borders of the Haversian systems. Where invasion of the new cartilage by the new forming bone can be demonstrated, the picture is exactly that of normal endochondral ossification (Figs. 7 and 8). The cartilage cells beyond the advancing lines of ossification show evidences of rapid proliferation, with subsequent degenerative changes and calcification of the matrix. The bone spicules contain marrow cavities rich in capillaries and are lined with endosteum. The borders of these spicules invade the calcified cartilage and in places incorporate shadows of matrix and lacunæ.

DISCUSSION.

Before discussing the new points brought out by the experiments it will be well to consider to what extent we have been able to corroborate the observations and views of previous investigators. Unlike Liek, who used a method similar to ours in studying the rabbit kidney, we did not find evidences of bone formation as early as 16 or 20 days after ligation of the renal vessels. This failure may have been due to differences in the rate of restoration of vascularity. Nor did we observe in the early stages of bone formation in the kidney the close juxtaposition of the bone and the calcium deposits noted by this

author. On the contrary, in our earliest stage the bone was located under the epithelium of the pelvis farthest from the calcified tubules. In all our kidneys prior to the time when the epithelium of the calices had grown out to the lime placques, no bone was found in the cortex.

As to the mode of transformation of connective tissue into bone, two distinct processes have been hitherto described by numerous writers. According to their views, either a hyaline connective tissue or scar tissue may be directly converted into bone, or calcified materials may be eroded with a formation of vascular areas containing young connective tissue cells, some of which take on the function of osteoblasts. As has been described previously in this paper, the histological pictures presented by our specimens probably represent three different methods of transformation. The first and most frequent is the accumulation of young fibroblasts in the area under the transitional epithelium to form a sort of membrane which lays down bone. The bone increases in size by progressive ossification with inclusion of cells of the periosteum-like membrane. Second in frequency is the type described by Liek, Rosenstein, Pearce, and others, in which there is a direct transformation of hyaline connective tissue into bone. This process takes place only in the presence of preexisting bone, and for this reason cannot be considered as a primary process. The third type, in which cells of the vascular granulating tissue which erodes calcium deposits become converted into osteoblasts, was observed only in one of the thirteen specimens showing bone formation.

The theory that heteroplastic bone is formed as a result of stimulation of young fibroblasts by lime salts rests mainly upon the recorded fact that the bone formation takes place in the immediate vicinity of the calcium deposits, since Liek and many others have been unable to confirm the statement made by Barth (16) that injection of calcium salts or implantation of dead bone will stimulate bone formation. In our series of calcium injection, the experimental data of which are omitted for the sake of brevity, no evidence of bone formation was observed as late as 50 days after the injection. In pathological calcification followed by ossification there may perhaps be other factors involved than the mere presence of calcium salts, and these as yet undetermined factors may be essential in the heteroplastic bone

formation. It seems evident that in a richly vascular tissue, cells a few millimeters away from the relatively insoluble salts would be bathed by a tissue fluid of practically the same composition as that found in any other part of the body. A close proximity between the bone and lime salts has not been found in our series. However, it is impossible to exclude the possibility that the young connective tissue cells may have received a stimulus from the calcium salts at the time they first migrated into the necrotic areas poor in blood supply, with the subsequent formation of bone when vascularity was restored and the area again comparatively free from calcium.

In the case of ossification in the cartilage series in which endochondral bone formation was observed, evidence points towards the stimulation of fibroblasts by almost direct contact with the calcified matrix of the cartilage. In the instances in which connection between cartilage and bone could not be established and the process of ossification resembled that in the kidney described as the first type, a stimulation by calcium may have played a part, since as Wells and Benson (17) have shown even the cartilages that do not ossify have some affinity for calcium and absorb the salts from the tissue fluids. It is not improbable that fibroblasts coming in contact with such cartilage might be caused to differentiate into osteoblasts under favorable conditions.

CONCLUSIONS.

1. Bone formation in the rabbit kidney with ligated vessels takes place (*a*) through the activity of young fibroblasts which accumulate to form a membrane-like structure; (*b*) subsequently by direct ossification of hyaline connective tissue in continuity with preformed bone; and (*c*) through erosion of lime placques by granulating tissue and laying down of lamellar bone by cells derived from fibroblasts.

2. Bone formation in the rabbit kidney begins not in direct contact with calcium deposits, but in the loose vascular connective tissue close under the transitional epithelium of the calices.

3. With autotransplanted ear cartilage of the rabbit there is an active new formation of cartilage in the connective tissue which surrounds the transplants, and the bone is formed by the fibroblasts from the perichondrium which erode and invade the calcified areas in this new cartilage.

4. The process of bone formation in the kidney is similar to that found in normal membranous ossification, while with the transplanted ear cartilage the process is identical with endochondral ossification.

We wish to express our appreciation to Dr. E. L. Opie for many courtesies extended to us in connection with this work.

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EXPLANATION OF PLATES.

PLATE 98.

FIG. 1. Bone formation in the rabbit kidney, showing a cell membrane between osseous and fibrous tissue. 35 days after the ligation of the vessels.

FIG. 2. Bone formation in the kidney, showing gradual transition from fibrous to osseous tissue. 35 day stage.

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[2] Since this article was submitted for publication, a paper by Mauclaire has appeared, summarizing the work on cartilage transplantation (Mauclaire, *Presse méd.*, 1920, xxviii, 545). He states that Zaworikine (1898), Saltykow (1900), and Giani (1911) observed ossification in transplanted ear cartilage.

FIG. 3. Bone formation in the kidney, showing continuity between osseous and fibrous tissue. Weigert-Van Gieson stain. 35 day stage.

FIG. 4. Megalocaryocyte engulfing myelocytes. From the marrow in the bone of a kidney removed 94 days after ligation.

PLATE 99.

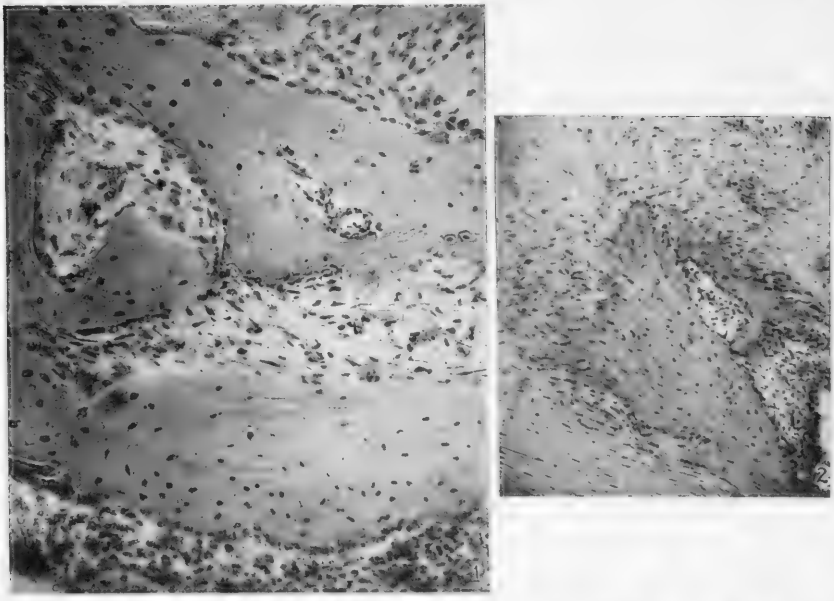
FIG. 5. Bone formation in the kidney, showing Haversian systems forming among eroded calcified tubules. 54 day stage.

FIG. 6. Bone formation with transplanted cartilage. Spicule of bone with marrow cavity and megalocaryocytes. 59 day stage.

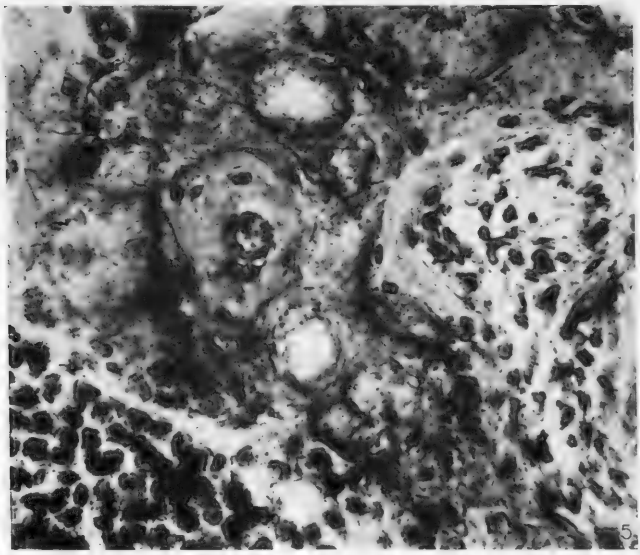
PLATE 100.

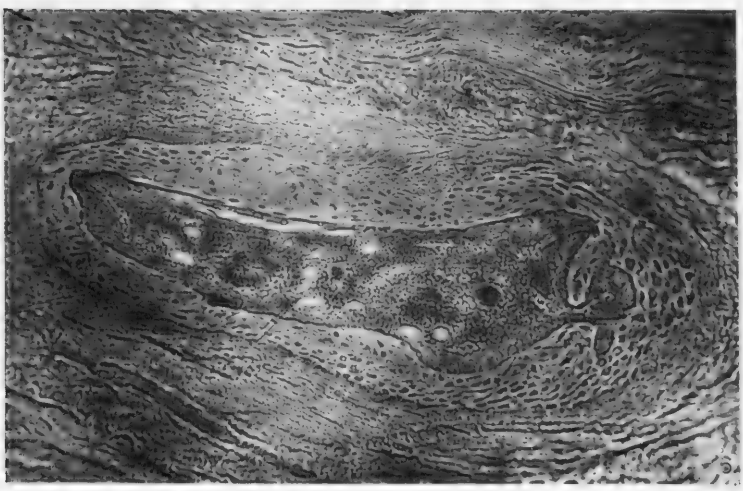
FIG. 7. Bone formation in an ear cartilage transplant. At the bottom are seen the hyaline matrix and cells of old cartilage. Above is new cartilage growth, with beginning invasion by capillaries and osteoblasts. 69 day stage.

FIG. 8. Bone formation in a cartilage transplant. On one side are seen hyaline cartilage cells. The rest of the figure shows Haversian systems forming under the perichondrium. 72 day stage.



(Asami and Dock: Heteroplastic bone formation.)





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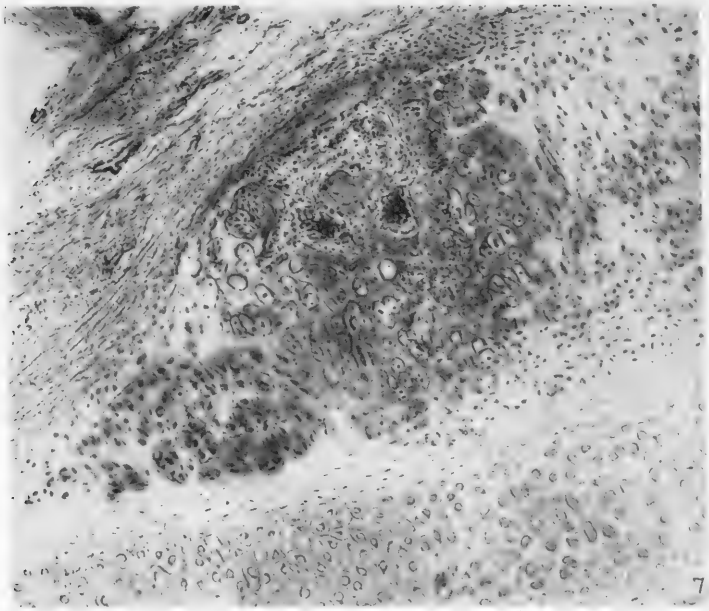

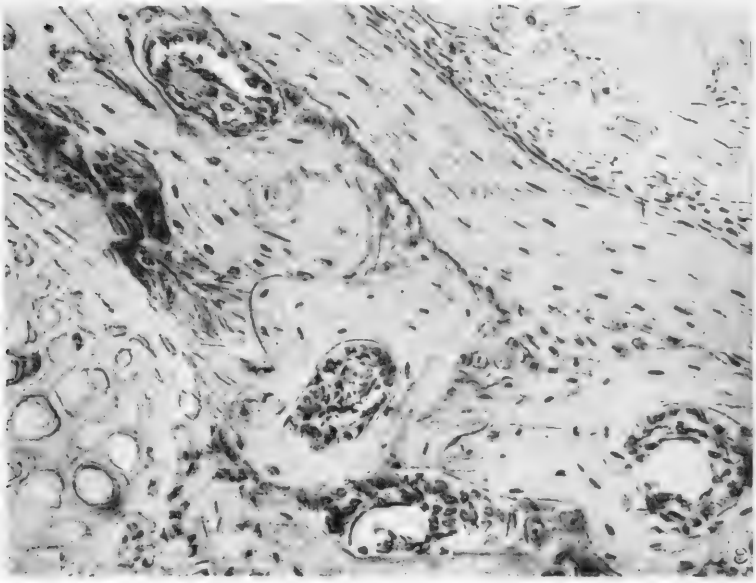

(Asami and Dock: Heteroplastic bone formation.)

SIMPLIFIED PRODUCTION OF ANTIMENINGOCOCCIC SERUM.

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Although a large amount of work in several countries has been done relative to the production of an effective antimeningococcic serum, the problems involved have not yet been wholly resolved. The increased demands arising from war conditions acted as a potent stimulus first to simplify manufacture and then to insure an effective product. This paper records primarily attempts to simplify the manufacture of an efficacious serum and deals incidentally with a number of still mooted questions regarding the antigenic properties of the meningococcus on which the production of such a serum largely rests.

It is now generally recognized that the meningococcus is not a simple, fixed antigenic entity but rather that the term meningococcus covers a class of closely related microorganisms, the distinguishing common characters of which relate to certain cultural and fermentative qualities and the power to set up in man particular forms of inflammation of the leptomeninges, while they differ markedly in their immunologic responses. Thus for identification the cultural properties are of first importance and for serum production the antigenic structure is paramount.

Ever since Dopter[1] first distinguished the immunologically distinct parameningococcus the classification of the meningococci has been under discussion and agreement has not yet been reached. Everyone admits the existence of two main groups called normal or regular meningococcus and parameningococcus; the disturbing factor is the

[1] Dopter, C., *Compt. rend. Soc. biol.*, 1909, lxvii, 74. Dopter and Pauron, *Compt. rend. Soc. biol.*, 1914, lxxvii, 231.

occurrence of intermediate cocci which resemble either the regular meningococcus or the parameningococcus but are immunologically less sharply defined than are the pure types of the main groups. This fact has led to the setting up of main and subsidiary types as, for example, in Gordon's classification which recognizes four types of meningococci.[2] But Gordon's classification has not received general acceptation and for the reason that observers cannot agree on the two subsidiary type strains.

These considerations have far more than theoretical interest, since experience has shown that a therapeutically effective antimeningococcic serum should possess wide capacities of immunologic activity as measured by the agglutinin content. Since the early work on the subject by Flexner and Jobling[3] and the later studies of Amoss and Wollstein,[4] it has been the custom to employ for the immunization of horses a large number of cultures including representatives of the regular, the para, and many intermediate strains of the meningococcus. The number of strains used in the antigen might reach 50 or more, depending upon the reaction of the serum with meningococci derived from the cerebrospinal fluid of many cases of epidemic meningitis. When the test with such a culture showed low agglutination titer the strain was added to those used for immunization. The purpose of this was to produce a serum with as wide an agglutination index as practicable.

It is obvious that this procedure implies an empirical method. In endeavoring to simplify the manufacture of antimeningococcic serum it seemed worth while to determine more precisely than had yet been done whether a wide agglutination index could be obtained from a small number of antigenically different strains. Several facts had already rendered it probable that something in this direction was achievable. For example, it had been noted that the immunity response in the horse was wider than in the rabbit and also that the longer the injections of certain fixed cultures were continued, the more inclusive the agglutinin content became. In addition to these essential data there

[2] Gordon, M. H., *Med. Research Com., Nat. Health Insurance, Special Rep. Series, No. 3*, 1917, 10.

[3] Flexner, S., and Jobling, J. W., *J. Exp. Med.*, 1908, x, 141.

[4] Amoss, H. L., and Wollstein, M., *J. Exp. Med.*, 1916, xxiii, 403.

was a still further important fact; namely, that approximately 80 per cent of all cases of epidemic meningitis arose from infection with the two type strains; that is, the regular meningococcus and the parameningococcus. Hence the production of an effective serum for this large proportion of cases of meningitis appeared to be relatively simple; the real problem was to make the serum effective against the 20 per cent of cases due to the highly variable subsidiary strains.

Fortunately, we possessed as a standard of comparison for the sera to be produced with a limited number of strains, samples of polyvalent sera, of established therapeutic efficacy, which had been produced at The Rockefeller Institute by the injection of 51 spinal cultures of the meningococcus selected in accordance with the method outlined above.[5] These 51 cultures were classified as follows: 10 were regular, 26 were para, and 15 were intermediate (subsidiary) strains of meningococcus.[6]

Horse Sera Produced with a Small Number of Strains.

When the experiments with horses with a smaller number of cultures of the meningococcus were begun in 1917 we had already observed that rabbits which were immunized with a single type strain over long periods of time yielded sera which contained agglutinins not only for that strain but also, in less amount, for heterologous strains including even those of the opposite type.

Use of Five Strains as Antigen.—The experiments described below were made on two horses (Nos. M 24 and M 25). Injections were begun with four cultures which were classed immunologically as

[5] The polyvalent serum issued for therapeutic purposes was composed of serum from at least three horses. Because of the variation in the response of different horses, it has been our practice to pool serum from several horses to insure a properly balanced product.

[6] In 1914 two strains of parameningococcus were brought from Dopter by a member of the staff of The Rockefeller Institute. It was on the basis of these strains that our classification was made. Recent comparison of our stock strains with sera and cultures lately obtained from England and France shows that our original classification is the reverse of the present accepted grouping. Conforming to general usage, we have revised our classification in this paper, so that the regular or normal group mentioned in previous papers from these laboratories is here designated as para and *vice versa*.

follows: Culture 1, para type strain; Culture 60, regular type strain; and Cultures 30 and 31 of different intermediate types. Serum obtained in early bleedings of these horses did not agglutinate Culture 38 (also an intermediate) which was then added to the antigen.

Experiment 1.—The method of immunization was as follows: After a glanders test and the subcutaneous injection of 1,500 units of antitetanic serum, Horses M 24 and M 25 were tested for sensitiveness to the meningococcus by the intravenous injection of minute doses (0.05 cc.) of a saline suspension (2.5 cc. to an agar slant culture). The immunizing injections were begun on November 29, 1917, living cultures being given in a total dose of 0.12 cc., and were continued according to the method described by Amoss and Wollstein.[4] Trial bleedings were made with Horse M 24 after 3, 10, and 14 months, and with Horse M 25 after 2, 10, and 14 months.

TABLE I.

Number of Stock Strains Agglutinated by the Serum of Horses M 24 and M 25 Immunized with Five Strains of Meningococcus.

Horse No.	Duration of immunization.	Agglutination test positive.	
		1:400 or higher.	1:100
	mos.		
M 24	3	35 of 51	50 of 51
" 24	10	43 " 60	59 " 60
" 24	14	51 " 56	56 " 56
" 25	2	35 " 58	40 " 58
" 25	10	32 " 60	58 " 60
" 25	14	47 " 56	56 " 56

In Table I the progress of the immunization is summarized according to the number of stock strains (cultures) which were agglutinated in a serum dilution of 1:100 and of 1:400 or higher. 51 of these stock strains were those used in the manufacture of the therapeutic polyvalent serum.

Table I indicates that by employing for purposes of immunization as few as five cultures, representing different strains, of the meningococcus, a serum is produced which shows considerable agglutinative capacity for as many as 51 selected stock cultures, including the

English type strains.[7] The titer of the serum for this large number of cultures equalled that previously required for a polyvalent serum prepared with a far larger number of cultures.

Use of Three Strains as Antigen.—In this experiment the strains were reduced to three, representing regular and para types.

Experiment 2.—The procedure with Horse M 31 was similar to that already described. The injections were begun on May 1, 1918, and trial bleedings were made after 5 and 9 months of immunization. Two regular cultures (Strains 60 and 79) and one para culture (Strain 85) were employed for the injection.

Table II summarizes the results obtained, which are the equivalent, for the same period of injection, of those obtained with the five strains as shown by Table I. Although thirteen of the stock cultures did not agglutinate in the 1:400 dilution after 9 months of immunization all were agglutinated in dilutions ranging from 1:50 to 1:200.

TABLE II.

Number of Stock Strains Agglutinated by the Serum of Horse M 31 Immunized with Three Strains of Meningococcus.

Duration of immunization.	Agglutination test positive.	
	1:400 or higher.	1:100
mos.		
5	32 of 50	47 of 50
9	43 " 56	55 " 56

Monovalent Horse Sera.

The total amount of meningococcic antigen which can be given a horse varies and is obviously limited. The use, therefore, of a single antigen might result in a greater antibody content for the homologous strain than could be obtained against the individual strains of a multiple antigen. Indeed, certain therapeutic monovalent sera have been prepared in this way by the Pasteur Institute and more recently by Gordon and others in England working under the Medical

[7] The English type strains were kindly supplied by Dr. Gordon.

Research Council.[8] The intent in both these instances was to fortify the therapeutic activity of the serum for treating cases of epidemic meningitis with type antisera after the type of infecting meningococcus had been determined.

It is not our intention in this place to discuss the practicability of this method of procedure or even to analyze the results thus far secured by the English investigators mentioned above. We would remark, however, that we are dubious as to the advisability of making a substitution of a monoserum for an active polyserum in view of the uncertainties and lack of uniformity which still surround the practical work of determining and distinguishing the so called types of meningococci. But as the data which follow show, the antigenic constitution of even type meningococci is such that a strictly monovalent serum is not produced when horses are injected with a type culture over a long period of time.

The experiments on monovalent sera were made in two ways: first, by injecting as many as three cultures of the same type of meningococcus, and second, by injecting a single type culture only.

Experiment 3.—Horse M 30, after the usual preliminary treatment, was first injected with suspensions of living parameningococci (Cultures 1, 4, and 36). After 6 and 10 months of immunization trial bleedings were made. The serum thus obtained was titrated against 56 stock cultures of meningococci with the results shown in Table III.

TABLE III.

Number of Stock Strains Agglutinated by the Serum of Horse M 30 Immunized with Three Strains of Parameningococcus.

Duration of immunization.	Agglutination test positive.	
	1:400 or higher.	1:100
mos.		
6	40 of 52	50 of 52
10	56 " 56	56 " 56

The results were striking and unexpected, and yet they confirmed certain tests which we had previously made on the variations in

[8] Hine, T. G. M., *Privy Council, Med. Research Council, Special Rep. Series. No. 50*, 1920, 176.

agglutinogenic activity exhibited by different strains of the meningococcus. It appears that in this respect separate strains of the meningococcus show a wide variation. It has been noted also that horses vary in their response (*cf.* Nos. M 24 and M 25, Table I).

The next step was to determine the effect of immunizing horses with single type strains of the regular meningococcus and the parameningococcus. For this purpose two horses were employed.

Experiment 4.—Horse M 32 was injected with a para culture (No. 1) and Horse M 33 with a regular culture (No. 60). The immunization was begun on December 5, 1918, and test bleedings were made 3 months later at a period still too early to show the final wide range of agglutinative capacities of the sera. However, the serum of Horse M 32 (para) agglutinated in dilutions of 1: 400 or higher 52 of the 56 stock cultures, and the serum of Horse M 33 (regular) agglutinated in the 1: 400 dilution or higher 51 of the 56 stock cultures.

These results raise certain very pertinent questions which cannot be answered offhand. We have dealt with certain aspects of these questions in a later section of this paper in connection with the changes which take place in the sera during storage and the response of the sera produced in different ways to selective absorption tests. We possess many observations which point to the greater efficacy of a truly polyvalent antimeningococcic serum in practice, and hence we do not accept, as yet, such an apparently polyvalent serum, arising from single type cultures, as being immunologically and therapeutically equivalent to the former. The results given show also that an immune horse serum cannot be used for classifying meningococci, and finally, that the so called monovalent sera prepared in France and England in the horse doubtless possessed agglutinative capacities far wider than is implied in their names.

Comparative Agglutinin Content of Monovalent and Polyvalent Sera.

The experiments on horses with single and with several strains of meningococci have shown that agglutinin formation is induced not only for the strain or strains injected but also for a wide number and diversity of other type and subsidiary strains. At first sight it may appear that the sera produced with single and with multiple strains are practically identical and could be substituted for each other in

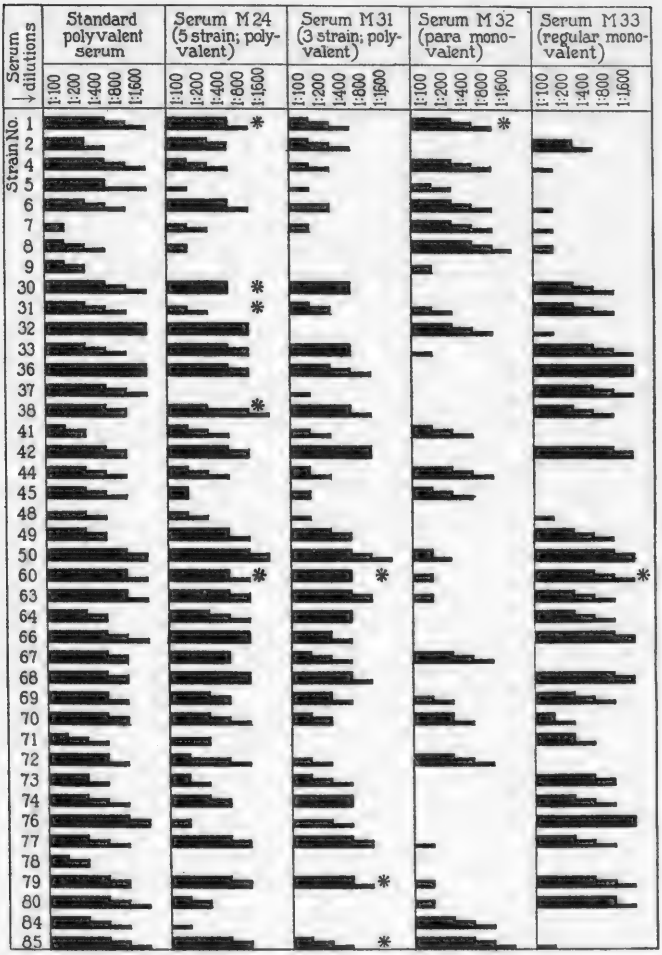
treatment. And yet this deduction would not be justified, as the following tests show. It is also in conflict with observations on the efficacy of widely polyvalent antimeningococcic serum known to contain specific agglutinins for the main and subsidiary strains of the meningococcus.

Effects of Storage.—The first tests to be described here relate to the comparative keeping qualities of the two kinds of sera. The samples of mono- and polyvalent horse sera were kept in the refrigerator in the dark at an approximate temperature of 4°C. for about 1 year. All the sera had been preserved with 0.15 per cent tricresol. The sera tested were derived from Horses M 32 (monovalent para), M 33 (monovalent regular), M 24 (regular, para, and three intermediates), and M 31 (three regular). The polyvalent serum used for comparison was obtained by pooling the serum of six horses, each immunized over a long period of time with 50 odd strains.

The tests of keeping qualities took into account only the agglutinins, which can be quantitatively determined. The titrations were made with suspensions of killed cultures which were uniform for all the tests.

The results of the tests are shown graphically in Text-fig. 1. While the polyvalent serum has fallen off but little during a year's storage and still agglutinates all of the 41 strains of meningococci employed, the two monovalent sera have lost agglutinating power for a considerable number of the strains (sixteen in the case of Horse M 32 (para type) and ten in Horse M 33 (regular type)). On the other hand, the three strain serum (Horse M 31) and especially the five strain (Horse M 24) approach in value the polyvalent serum. The serum of Horse M 24, in a dilution of at least 1: 50, agglutinated 39 of the 41 strains.

The chart brings out the important fact that the common or secondary agglutinins are the first to disappear from the serum and that specific agglutinins for homologous strains are reasonably stable during storage. This is a point of capital distinction and may well prove to be the determining factor in respect to the manufacture as well as to the therapeutic efficacy of the various sera. The striking difference as shown by the pooled polyvalent serum relates to its inclusiveness for essentially all the strains employed in the test. It



TEXT-FIG. 1. An agglutination test with five sera after storage for 1 year or longer in the ice box. In four of the sera asterisks indicate homologous strains.

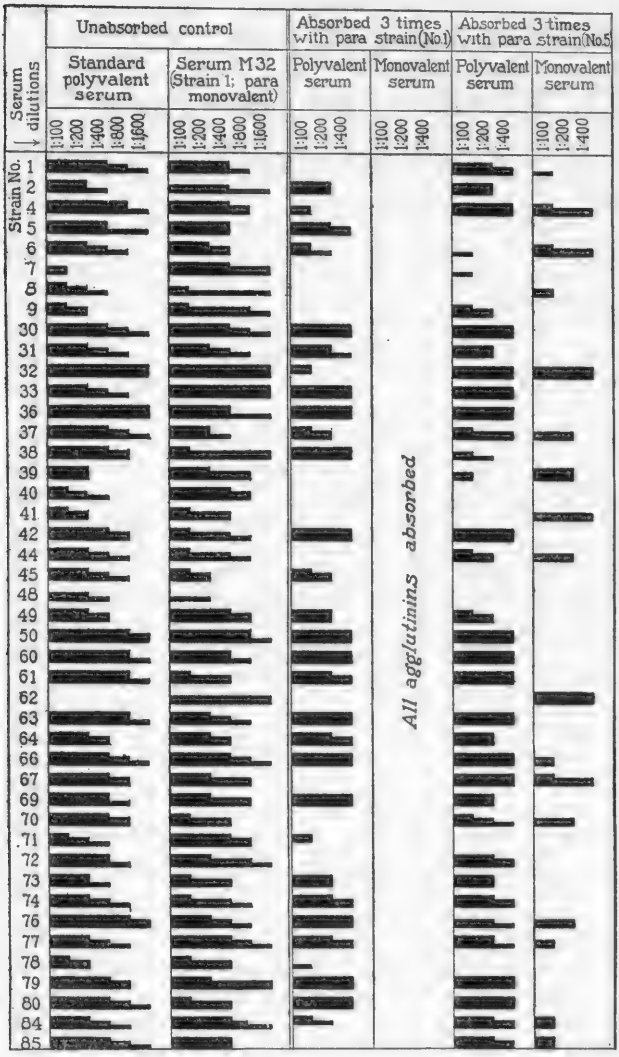
Complete agglutination in any dilution is represented by a broad black band and indicates the flocculation of all organisms, leaving a clear supernatant fluid, even after shaking. Incomplete agglutination, represented by a band of medium width, indicates the flocculation of almost all the organisms, the supernatant fluid remaining hazy after shaking. Partial agglutination, represented by a narrow band, indicates the presence of distinct flocculi which do not disappear on shaking, although the supernatant fluid is cloudy with unagglutinated organisms.

is obvious that as the monovalent sera lose their secondary (or common) agglutinins they become more suitable for use in type diagnosis, and *pari passu*, more unsuitable for therapeutic purposes. One further matter may be pointed out. The standard polyvalent serum was prepared by pooling the sera derived from six horses. The sera prepared with three and five strains respectively came each from one horse. Reasoning by analogy and taking into account the response of different horses to injections of various strains of meningococcus, for example, Horses M 24 and M 25, it is quite possible that the pooling of sera prepared from a small number of type strains might yield an enduring and still more inclusive serum.

Effects on Absorption of Agglutinin.—The differences in deterioration on storage point to a fundamental difference in the monovalent as compared with the polyvalent sera which is confirmed by the test for absorption of agglutinin as is shown graphically in Text-fig. 2.

In Columns 1 and 2 are shown the control agglutination tests made with a 14 month sample of the pooled polyvalent serum and a fresh sample of monovalent para serum (Horse M 32) before absorption with killed cultures of Strains 1 (the homologous para strain) and 5 (also of para type). After triple absorption under carefully controlled conditions, it was found that the single strains had removed all the agglutinins with which they could react, since further absorption did not reduce significantly the agglutinins remaining in the serum.

Text-fig. 2 shows that Strain 1, the homologous strain, exhausted the monovalent serum completely, but was unable to exhaust the polyvalent serum, leaving agglutinins with which 30 of the 44 test strains were able to react. Similarly, while Strain 5, another strain of the same type, removed from the monovalent serum all the agglutinins with which 27 of the 44 test strains had reacted in the control tests, a similar treatment of the polyvalent serum left available agglutinins for all but 9 of the 44 test strains. This experiment emphasizes the difference in the inherent character of the polyvalent and the monovalent serum.


Unabsorbed control
Absorbed 3 times with para strain(No.1)
Absorbed 3 times with para strain(No.5)
Standard polyvalent serum
Serum M32 (Strain 1; para monovalent)
Polyvalent serum
Monovalent serum
Polyvalent serum
Monovalent serum
Serum dilutions →
1:100 1:200 1:400 1:800 1:1600
1:100 1:200 1:400 1:800 1:1600
1:100 1:200 1:400
1:100 1:200 1:400
1:100 1:200 1:400
1:100 1:200 1:400
Strain No.
1 2 4 5 6 7 8 9 30 31 32 33 36 37 38 39 40 41 42 44 45 48 49 50 60 61 62 63 64 66 67 69 70 71 72 73 74 76 77 78 79 80 84 85
All agglutinins absorbed

TEXT-FIG. 2. A comparison by agglutination of standard polyvalent serum and of para monovalent serum before and after absorption three times with a heavy suspension of Strain 1 or 5.

Employment of Killed Cultures.

The preparation of therapeutic meningococcic serum with living antigen is laborious and difficult. Because of the rapidity with which the meningococcus dies in artificial cultures, frequent transfers on serum media must be made, and the cultures to be used for the immunizing injections must be freshly prepared each time in a plain agar medium. The question arises, therefore, whether the practical operations cannot be simplified by the employment, at least over certain periods, of killed cultures of the meningococcus so prepared as to prevent the autolysis which tends to destroy the antigenic properties. We have not carried out an exhaustive study of this subject but from many tests on rabbits and the following experiment with Horse M 28, we are of the opinion that the subject is worthy of thorough investigation.

Experiment 5.—Because of the rapidity with which the meningococcus autolyzes in cultures, growths in plain agar in Blake bottles, incubated at 37°C. for 8 to 10 hours, were employed. The surface growths were washed off with 20 cc. of isotonic saline solution and the heavy suspensions were quickly heated in a water bath to 65°C. to destroy the autolytic ferment. Test for viability was made and 0.35 per cent tricresol added. The suspension was kept in the refrigerator.

Horse M 28, after the usual preliminary treatment, was injected, beginning January 4, 1918, with suspensions of killed cultures of the same strains that were used in the immunization of Horses M 24 and M 25; *viz.*, No. 1 (para), No. 60 (regular), and Nos. 30, 31, and 38 (intermediates). The test serum bleedings were made after 7 and 12 months of immunization with the results shown in Table IV.

TABLE IV.

Number of Stock Strains Agglutinated by the Serum of Horse M 28 Immunized with Killed Cultures of Five Strains.

Duration of immunization.	Agglutination test positive.	
	1:400 or higher.	1:100
mos.		
7	46 of 60	59 of 60
12	53 " 56	56 " 56

This single experiment may be taken merely to indicate that as far as the agglutinin response is concerned, killed cultures of me-

ningococci can be used for immunizing horses. It will be patent from all the circumstances and from what has been stated above, that this fact is not regarded as tantamount to the conclusion that a therapeutically active and efficacious antimeningococcic serum can be produced in this way. We should, indeed, be willing to go no further at the present time than to propose that the suspended killed cultures be kept on hand to be used on occasion in place of the suspended live cultures when for any reason circumstances make it impossible to inject the latter. During the war when antimeningococcic serum was being produced at The Rockefeller Institute under great pressure, we never relied on the killed cultures alone and, at most, only occasionally alternated the injection of the killed and the living cultures in the routine manufacture of antimeningococcic serum. We still believe in the practice of employing live cultures and also of cultures freshly isolated from cases of epidemic meningitis.

The power of stored killed cultures, preserved with tricresol, to induce agglutinin formation was tested in one instance in rabbits. The killed cultures had been in the refrigerator for periods of 8 and 14 months. The rabbits tolerated the usual doses. One rabbit injected with a killed regular strain gave a serum of 1: 1,600 titer, and another injected with a killed para strain gave a titer of 1: 800 against freshly prepared killed cultures. The stored killed suspensions were agglutinated in other immune rabbit sera in high dilutions. As a conservative routine, however, the killed cultures employed occasionally for injection and for agglutination were not used after 3 months storage.

SUMMARY AND CONCLUSIONS.

In an attempt to simplify the manufacture of an efficacious antimeningococcus serum an experimental study has been made of a number of sera produced with a few or with single strains of meningococcus, the therapeutic polyvalent serum produced at The Rockefeller Institute with more than 50 strains being used as a standard of comparison.

It was found that horses injected with an antigen limited to five, three, or even one strain yielded sera with a range of agglutinins covering in high dilution practically all the stock strains used in pro-

ducing the polyvalent serum. These sera appeared to equal the polyvalent serum in range and titer of agglutinins, but on further examination fundamental differences were found. Storage for a year had little effect upon the titer and inclusiveness of the polyvalent serum, whereas the monovalent serum had fallen off greatly, especially in regard to secondary or subsidiary agglutinins, so that only a comparatively small number of stock strains was still agglutinated. The serum made with five strains, a regular, a para, and three intermediate meningococci, approached the polyvalent serum in keeping qualities and still agglutinated at the end of this period 39 of the 41 strains tested.

Absorption tests also brought out inherent differences in the nature of the polyvalent and the monovalent sera which had appeared to be practically identical in simple agglutination tests. The homologous strain on triple absorption was able to exhaust the monovalent serum completely, but was unable to remove from the polyvalent serum agglutinins to which 30 of 44 different strains were able to react. Absorption with another single strain of the same type removed from the monovalent serum agglutinins for a majority of the test strains but left the polyvalent serum relatively unaffected.

It is comparatively easy to produce a serum effective against about 80 per cent of the spinal strains of meningococci encountered. Deficiencies in our knowledge of the antigenic capacities of the meningococcus have led to the more or less empirical use of a large number of cultures in the preparation of a serum effective against the remaining 20 per cent of the strains. How far the number of the latter in the antigen may be reduced without restricting the efficacy of the serum remains yet to be determined. However, the experimental evidence recorded here apparently does not favor the use of an antigen limited to one or too few strains. For example, three or five selected strains produced a serum which agglutinated practically all the strains against which it was tested. But in view of the many observations which point to the greater therapeutic efficacy of a serum made with a larger number of strains we would not as yet advocate a serum prepared with too limited antigens even though it contains at first a wide range of agglutinins.

It has been brought out that a monovalent serum contains, in addition to specific agglutinins, a wide range of common or secondary agglutinins which tend to disappear during storage. The difference between specific and secondary agglutinins is not apparent in simple agglutination tests, but is revealed by absorption tests. It is probable that in a serum prepared with a few strains the same condition exists, whereas in a serum produced with a large number of strains the agglutinins are mainly specific as contrasted with the fact that most of them are secondary in the serum produced with few strains. The question whether secondary agglutinins are therapeutically equivalent to primary or specific agglutinins requires further study.

We wish to acknowledge our indebtedness to Dr. J. H. Brown and Dr. R. B. Little, of the Department of Animal Pathology of The Rockefeller Institute, for their cooperation.

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